REMARKS

Claims 1-43 are pending in the above-identified patent application. Claims 1, 12, 21, 33, 38, 40 and 43 are amended herein. Claims 2, 3 and 41 are canceled by this Amendment. Claims 23, 24, 28 and 29 are withdrawn.

1. Restriction

The Applicants confirm election of Group I, claims 1-39 and 43. Claim 41 has been canceled, and claim 42 has been withdrawn. Applicants have amended claim 40 with the understanding that rejoinder of this claim may be made in the event that product base claim 1 is ultimately determined to be allowable.

Group I claims were further restricted by the Examiner to A = arylene, E = N, X = $-CR^aR^b-$, each R^1 is as claimed except for heteroalkyl, and R^{10} is as claimed except for heteroaryl or heterocyclyl. Claims 1, 38 and 43 have been amended to remove non-elected subject matter in accordance with this restriction. Claims 23, 24, 28, 29, which are directed to non-elected subject matter, have been withdrawn.

With regard to rejoinder of method of treatment claims 40-42, the Examiner required election of a single indication (memory disorder, manic depression, Huntington's Disease, Alzheimer's disease, etc.) in the event that claims 40-42 are rejoined for examination. During the telephone conversation with the Examiner on September 10, 2004, Applicants' traversed the Examiner's restriction of Applicants method of treatment claims according to different central nervous system (CNS) indications (i.e., Applicants' election of "memory disorder" was made with traverse). The recited method of treating various CNS indications recited in claim 41 (psychoses, schizophrenia, manic depressions, neurological disorders, memory disorders, attention deficit disorder, Parkinson's disease, amyotrophic lateral sclerosis, Alzheimer's disease and Huntington's disease) cannot reasonably represent eleven separate and indistinct inventions as suggested by the Examiner, and Applicants' believe that restriction along these lines is not proper.

"Independent" as defined in MPEP §802.01 requires that there is "no disclosed relationship between the two or more subjects disclosed, that is, they are unconnected in

design, operation or effect ...". The Examiner points out, on page 12 of the Office Action, that the pathology of various CNS diseases is recognized in the prior art as being potentially linked to the 5-HT6 receptor. Treatment of these various CNS indications in the manner recited by Applicants would involve use of the same product from base claim 1 (common design), by administration to a subject (common mode of operation) to modulate the 5-HT6 receptor (common effect). Accordingly, Applicants respectfully believe that the methods of treating these various 5-HT6 mediated CNS indications with Applicants' recited product cannot reasonably be "independent".

The term "distinct" (MPEP §802.01) means that two or more related subjects "are capable of separate manufacture, use or sale as claimed, AND ARE PATENTABLE (novel and unobvious) OVER EACH OTHER" (original emphasis from MPEP). The Examiner, by making the restriction presented in the Office Action, indicates that a claim for method for treating memory disorders using the compound of Applicants' claim 1, would be patentably distinct from, for example, a method for treating Alzheimer's disease (or Parkinson's disease, Huntington's disease, etc) using the same compound.

If an unrelated party had previously disclosed (published) a method for use of a particular 5-HT6 receptor antagonist for treatment of memory disorders, would a subsequent claim by Applicants for a method of using the same 5-HT6 receptor antagonist for treatment of Alzheimer's disease (or other CNS indication known to be related to the 5-HT6 receptor) be patentable over such a disclosure? The Applicants believe not. There are numerous references in the prior art linking the 5-HT6 receptor to each of Applicants' recited CNS indications (psychoses, schizophrenia, manic depressions, neurological disorders, memory disorders, attention deficit disorder, Parkinson's disease, amyotrophic lateral sclerosis, Alzheimer's disease and Huntington's disease). For example, the undersigned attorney carried out an internet search using the "Google" search engine (on the date below) for "5-HT6" and "memory disorder" and received 258 "hits", many of which represent scientific publications. A similar search for "5-HT6" and "Alzheimer's" resulted in 302 "hits", again many of which represent scientific publications. These publications are presumably well known to persons skilled

in the art, and Applicants believe that skilled persons would find it obvious to use a known 5-HT6 receptor antagonist for treatment of an indication known to be linked to the 5-HT6 receptor. Applicants respectfully submit that a method of treating psychoses, a method of treating manic depressions, a method of treating neurological disorders, etc., using the product of claim 1, are not patentably distinct and thus are not "distinct" within the meaning of 35 USC §121. Accordingly, Applicants believe that the Examiner's restriction of the various methods within Group II is not proper.

Applicants further note that, in the event that the compound of base claim 1 is allowable, the use of such a compound for any of Applicants' recited indications would per se be novel and non-obvious over the prior art. Thus, no additional search and examination with respect to the prior art would be required, and consideration of the recited indications together would not present an undue burden.

2. Claims Subject To Objection

The Examiner objected to claims 1-22, 25-27, 30-39 and 43 were objected to on the grounds of non-elected subject matter. Claims 1, 38 and 43 have been amended as noted above to conform to Applicants' elected subject matter. Claims 2-22, 25-27, 30-37 and 39 depend directly or indirectly from amended claim 1 and are believed to recite only elected subject matter.

3. Rejection of Claim 39/40 Under 35 USC §112, First Paragraph

The Examiner rejected claim 39 under 35 USC §112, first paragraph as not being enabled by Applicants' specification for treatment of all memory disorders (while Office Action indicates claim 39, Applicants believe that the Examiner is addressing claim 40 in this rejection). The Examiner indicated that, in the state of the prior art, 5-hydroxytryptamine is recognized as having a potential a role in the pathology Parkinson's disease, Huntington's disease, anxiety, manic depression and psychoses, but that the efficicacy of 5-hydroxytryptamine modulators on memory disorders has not been established in the art.

The Applicants respectfully disagree. The link between the 5-HT6 receptor and cognitive/memory disorders is well established, and the effectiveness of 5-HT6 receptor antagonists in memory enhancement has been demonstrated. At least three selective 5-HT6 receptor antagonists are currently undergoing clinical trials for treatment of memory dysfunction and Alzheimer's disease: GSK 742457 (Glaxo Smith Klein, Phase I); M100907B (Aventis Pharmaceutical, Phase I); and SGS518 (Saegis Pharmaceutical, Phase I). The Glaxo Smith Kline selective 5-HT6 antagonist SB-271046 has recently been dropped from clinical trials.

The link between treatment of memory disorders and the 5-HT6 receptor is well established in the Scientific literature. Enhanced retention of spatial learning in rats following administration of a 5-HT6 antagonist has been specifically shown by Woolley et al., "A Role for 5-HT6 Receptors in Retention of Spatial Learning in the Morris Water Maze", Neuropharmacology 41, 210-219 (**2001**). The memory enhancing effects of 5-HT6 antagonists in the water maze test were also confirmed by Rogers et al., "5-HT6 Receptor Antagonists Enhance Retention of a Water Maze Task in the Rat", Psychopharmacology 158, 114-119 (**2001**). Additional exemplary publications linking 5-HT6 antagonists to treatment of memory disorders are: Riemer et al., "Influence of the 5-HT6 Receptor on Acetylcholine Release in the Cortex ", J. Med. Chem. 46, 1273-1276 (**2003**); Dawson et al., "*In Vivo* Effects of the 5-HT6 antagonist SB-271046 on Striatal and Frontal Cortex Extracellular Concentrations of Noradrenaline, Dopamine, 5-HT, Glutamate and Aspartate", British J. Pharmacology 130, 23-26 (**2000**); Branchek et al., "5-HT6 Receptors as Emerging Targets for Drug Discovery", Ann. Rev. Pharmacol. Toxicol. 40, 319-334 (see p. 329 in particular) (**2000**); Woolley et al., "Reversal of a Cholinergic-Induced Deficit in a Rodent Model of Recognition Memory by the Selective 5-HT6 receptor Antagonist Ro 046790", Psychopharmacology 170, 358-367 (**2003**); and Dawson et al., "5-HT6 Receptor Antagonist SB-271046 Selectively Enhances Excitatory Neurotransmission in the Rat Frontal Cortex and Hippocampus", Neuropsychopharmacology 25 No. 5, 662-668 (**2001**). Copies of these publications (which predate Applicants' filing date) are submitted herewith.

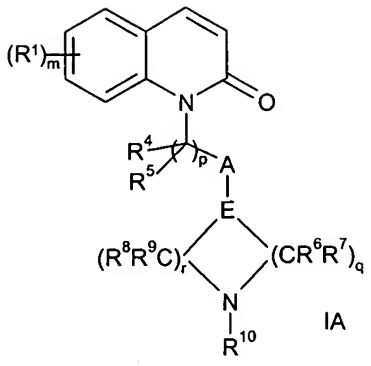
Accordingly, Applicants respectfully submit that the use of 5-HT6 receptor antagonists for the treatment of memory disorders has been established in the art, and skilled persons can reasonably test such compounds for treatment of memory disorder indications without undue experimentation.

Applicants have amended claim 40 to more specifically recite memory disorders and the various indications noted by the Examiner as being potentially linked to the 5-HT6 receptor. Claim 40 has been further amended to recited "5-HT6 antagonist mediated diseases" in order to limit the types of memory disorders treatable to those associated with the 5-HT6 receptor. Applicants believe that claim 40 as amended meets the criteria of 35 USC §112.

4. Claim Rejections Under 35 USC §112, Second Paragraph

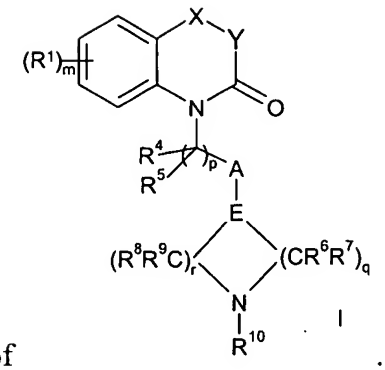
Claims 1-22, 25-27 and 43 were rejected under 35 USC §112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject mater which Applicants regard as the invention.

In claim 1, the Examiner indicated that the phrase "X and Y together form an alkenylene group is indefinite and unclear. On page 5 of Applicants' specification, "alkenylene" is defined as "a linear unsaturated divalent hydrocarbon radical of two to six carbon atoms or a branched saturated divalent hydrocarbon radical of three to six carbon atoms, e.g., ethenylene (-CH=CH-), 2,2-dimethylethenylene, propenylene, 2-methylpropenylene, butenylene, pentenylene, and the like. As recited in claim 1, X and Y can form a divalent, unsaturated radical such as ethenylene (-CH=CH-). If X and Y form an ethenylene, the formula of claim 1 would be:



IA

, instead of



I

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See, for example, compounds 23-27 of Table 1 of Applicants' specification, as well as the formulas in claims 33 and 34. Preserving the structure and valencies shown in formula I, there is only one way in which ethenylene could be introduced into formula I, and would result in formula IA above. Formation of another ring (i.e., tricyclic structure) would not be possible within the constraints recited in claim 1.

Applicants have amended claims 1 and 43 to more specifically recite " X and Y together form **a -CH=CH-** ~~**an alkenylene**~~ group". Applicants believe that this amended language more particularly points out and distinctly claims the subject matter of Applicants' invention. Support for this amended language is found at paragraph 14, in compounds 23-27 of Table 1, and elsewhere in Applicants' disclosure. Applicants have also amended claim 33 to change its dependency to claim 1 from intervening claim 32, in order to conform to this amended language.

The Examiner stated that "optionally substituted phenylene" in claims 7 and 16, and the recitation of "halophenylene, haloalkylphenylene, alkylphenylene, alkoxyphenylene or alkylenedioxyphenylene" in claims 12 and 21 lack antecedent basis. Applicants have amended claim 1 to recite "optionally substituted arylene" instead of merely "arylene", in order to provide antecedent basis for "optionally substituted phenyl" and the specific substituted phenyls noted by the Examiner. Support for this amended language is found in particular in paragraphs 17, 18 and 31(pages 5 and 7) of Applicants' specification.

Applicants believe that, in view of the amendments noted above, Applicants claims meet the criteria of 35 USC §112.

5. Other Items

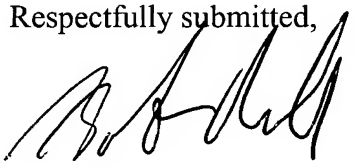
Applicants have amended claims 12 and 21 to include "cyclopentyloxy" within the markush group. Support for this amended language is found at compound 6 of table 1 of Applicants' disclosure.

CONCLUSION

The Applicants respectfully believe that all claims pending in the above-identified case are now in condition for allowance. If the Examiner believes a telephone conference would expedite prosecution of this application, please telephone the undersigned at 650-354-7540.

No fees should be due. However, in the event it is determined that a fee is due, please charge same to Deposit Account No. 18-1700.

Respectfully submitted,



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ORIGINAL INVESTIGATION

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5-HT_6 receptor antagonists enhance retention of a water maze task in the rat

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Abstract *Rationale:* 5-HT_6 receptors are predominantly located in the brain and may be involved in cognitive processes. The aim of this study was to assess the effects of two potent and selective 5-HT_6 receptor antagonists, SB-271046-A and SB-357134-A, on learning and memory in the rat. *Methods:* Spatial learning and memory was assessed by testing the effects of SB-271046-A and SB-357134-A on acquisition and retention of a water maze task. *Results:* In the water maze, administration of SB-271046-A or SB-357134-A (3 or 10 mg/kg) had no effect on learning per se. At 10 mg/kg, however, both compounds produced a significant improvement in retention of a previously learned platform position when tested 7 days after training. By contrast, the acetylcholinesterase inhibitor, Aricept (donepezil, 0.1, 0.3 mg/kg PO) had no effect in this task. *Conclusions:* This study demonstrates that systemic administration of SB-271046-A and SB-357134-A produces improvements in retention of a water maze task in the rat. These data indicate that 5-HT_6 receptor antagonism may be involved in cognitive function.

Keywords 5-HT_6 receptor · SB-271046-A · SB-357134-A · Aricept · Water maze · Learning and memory · Rat

Introduction

The 5-hydroxytryptamine (5-HT) receptor family comprises at least 14 distinct subtypes which mediate the effects of this modulatory neurotransmitter in man (Hoyer and Martin 1997). The 5-HT_6 receptor is the most recent addition to this family and the human 5-HT_6 receptor was first cloned by Kohen et al. (1996) Since this time, the 5-HT_6 receptor has been the focus of increasing interest as an emerging target for drug discovery (Branchek and Blackburn 2000). Until recently, no selective pharmacological tools have been available to characterise the role of the 5-HT_6 receptor in vivo, and functional studies were restricted to the use of antisense oligonucleotides which reduce the expression of the receptor (Bourson et al. 1995; Sleight et al. 1996). These studies demonstrated that administration of antisense oligonucleotides directed at 5-HT_6 receptor mRNA elicited stretching and yawning behaviours, which were blocked by atropine but not by haloperidol. Thus 5-HT, acting via 5-HT_6 receptors, may mediate an inhibitory tone on cholinergic neurones. Further studies with Ro 04-6790, a selective 5-HT_6 receptor antagonist, have demonstrated an increase in stretching behaviour (Bentley et al. 1999) and reversal of scopolamine-induced rotation in 6-OHDA-lesioned rats (Bourson et al. 1998). This supports conclusions from the antisense studies that the 5-HT_6 receptor is involved in the control of acetylcholine neurotransmission in the rat brain. More recently, an in vivo microdialysis study has suggested that excitatory amino acid neurotransmission is involved in the function of 5-HT_6 receptor antagonism and it has been demonstrated that the potent and selective 5-HT_6 receptor antagonist, SB-271046, increases extracellular levels of glutamate and aspartate in the frontal cortex of rats (Dawson et al. 2000).

Alzheimer's disease is characterised by complex pathology including marked decline in the cholinergic system which correlates strongly with the decline of cognitive ability (Coyle et al. 1983). In addition, animal studies have suggested that acetylcholine plays an important role in learning and memory and cumulative evidence indicates that the 5-HT system plays a modulatory role in cognitive processes, particularly in learning and memory (Roman and Marchetti 1998). Therefore, selective 5-HT_6 receptor antagonists that may be involved in modulation of the cholinergic system may also enhance cognitive processes in the rat and provide symptomatic treatment for dementia.

SB-271046-A (5-chloro-3-methylbenzo[b]thiophene-2-sulfonic acid (4-methoxy-3-piperazin-1-ylphenyl) amide

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hydrochloride) (Bromidge et al. 1999; Routledge et al. 2000) and SB-357134-A (*N*-(2,5-dibromo-3-fluorophenyl)-4-methoxy-3-piperazin-1-ylbenzenesulfonamide hydrochloride) (Bromidge et al. 2000) have been characterised as potent and selective 5-HT_6 receptor antagonists. The binding profile of these compounds indicated a pKi of 8.9 and 8.5, respectively, at the human 5-HT_6 receptor with greater than 200-fold selectivity over 51 receptors, ion channels and enzymes. SB-271046-A and SB-357134-A are orally bioavailable and, in a functional in vitro model using human cloned receptors expressed in HeLa cells, have been demonstrated to be competitive antagonists of the 5-HT_6 receptor with a pA_2 of 9.1 and 8.7, respectively. In addition, SB-271046-A has been demonstrated to increase seizure threshold in the rat maximal electroshock seizure threshold test (Routledge et al. 2000). The time-course of activity in this pharmacodynamic model was established with both SB-271046-A and SB-357134-A. SB-271046-A had a rapid onset of action (<30 min) reaching a maximum effect at 4 h post-dose, and maintained biological activity for at least 21 h (Routledge et al. 2000). SB-357134-A had a slightly longer onset of action (<60 min), reaching a maximum effect at 6 h post-dose (unpublished data). These data were used to select a pre-treatment time of 2 h for SB-271046-A and 4 h for SB-357134-A.

In the present study, we have investigated the effect of systemic administration of SB-271046-A and SB-357134-A in a water maze spatial learning and memory task (Morris 1981), using young, healthy unimpaired rats. For comparison, the acetylcholinesterase inhibitor, Aricept (donepezil) was also tested in this water maze procedure.

Materials and methods

This work was conducted in compliance with the Home Office Guidance on the operation of the Animals (Scientific Procedures) Act 1986, and was reviewed and approved by the SmithKline Beecham Procedures Review Panel.

Water maze

Animals

Naive, young, healthy, male Lister-Hooded rats (225–300 g, Harlan Olac, Bicester, UK) were housed in groups of four in a controlled environment (temperature=21.9°C±1°C; humidity=53±2%) and maintained on a 12-h light/dark cycle with lights on at 07:00 h with food and water available ad libitum.

Experimental design

The water maze (Morris 1981, 1984) was used to assess spatial learning and memory and details of the maze used in this study have been reported previously (Rogers and Hunter 1997). The water maze consisted of a white Perspex pool (200 cm diameter) filled with water made opaque white by the addition of an odourless latex compound (Opacifier E308, Morton Thiokol, UK). The pool circumference was arbitrarily marked with four start positions, (N, S, E, W) and divided into four virtual quadrants. The platform (a 15 cm Perspex disk) was anchored below the surface, and was therefore invisible to the rat swimming in the water. A video camera was positioned directly above the pool and was connected to an image analyser (HVS Image, Hampton, UK) which transformed the normal video image into a picture of high-contrast edges. A PC calculated measurements of latency, path length, number of platform crossings and percent time spent in each quadrant for each trial. Acquisition of the platform position by each rat was quantified by analysis of latency to find the platform, the path length of each trial during the training procedure, and percentage time spent in the platform quadrant during the probe trial (transfer test). Retention of the task was assessed at 4, 7, or 10 days after training in which each animal received a single 60-s transfer test with the platform removed from the pool, and percent time in the platform quadrant was calculated. Swimming speed was determined by analysis of the path length during the transfer tests, which had a fixed trial duration of 60 s.

Each rat received four consecutive trials on day 1 of training and six trials on days 2–4. On day 5, each rat received six trials followed by a probe trial (transfer test). At the beginning of each trial the rat was lowered gently feet first into the water, facing the wall at a start position (N, S, E, W) which was pre-determined randomly. A remote control was used to activate the computer and the rat was allowed to swim for 60 s to find the platform. If the platform was found during this time, the trial was stopped, the recording terminated using the remote control, and the rat left on the platform for 10 s. If the platform was not found during this time, the rat was retrieved quickly from the water and placed on the platform for 10 s.

SB-271046-A was suspended in half final volume of distilled water and made up to final volume with 2% methyl cellulose (Sigma) in water to give a dose-volume of 2 ml/kg in 1% methyl cellulose vehicle. In the first experiment, 30 rats received SB-271046-A (0, 3 or 10 mg/kg PO, n=10) 2 h prior to test on each of 5 consecutive training days, and prior to a retention test at 4, 7 and 10 days after training. Following analysis of the data from this experiment (see Results), it was observed that there was no further decline in performance in the control group after the 7-day retention test and therefore all subsequent experiments were carried out without the 10-day retention test. In the second experiment, 48 rats received either vehicle or SB-271046-A (3 or 10 mg/kg PO, n=16) 2 h prior to test on each of 5 training days, and two retention tests at 4 and 7 days after training.

SB-357134-A was suspended in half final volume of distilled water and made up to final volume with 2% methyl cellulose (Sigma) in water to give a dose-volume of 2 ml/kg in 1% methyl cellulose vehicle. Forty-eight rats received either vehicle or SB-357134-A (3 or 10 mg/kg PO, n=16), 4 h prior to test on each of 5 training days, and two retention tests at 4 and 7 days after training.

Aricept (Tocris Cookson, Southampton, UK) was dissolved in distilled water vehicle. Forty-eight rats received either vehicle or Aricept (0.3 or 1 mg/kg PO, n=16) 30 min prior to test on each of 5 training days, and two retention tests at 4 and 7 days after training.

Statistics

Data for latency, path length and percentage time in the platform quadrant were analysed by repeated measures ANOVA with post-hoc testing using Scheffe multiple comparisons. All statistical analyses were carried out using Statistica for Windows (StatSoft Inc., Tulsa, USA, Release 5.1, 97 Edition).

Results

SB-271046-A

In the first experiment, all rats performed this task well and learnt the position of the platform during the acquisi-

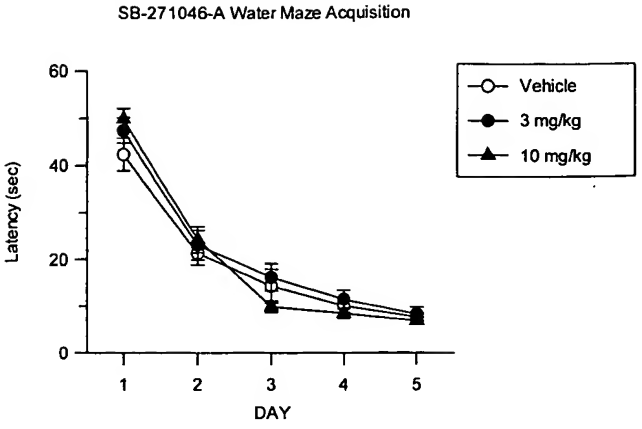

Fig. 1 Effect of SB-271046-A (3 and 10 mg/kg) on water maze acquisition. Data are presented as mean±SEM of latency (s) to find the platform (blocked across day) for each of 5 training days (n=10)

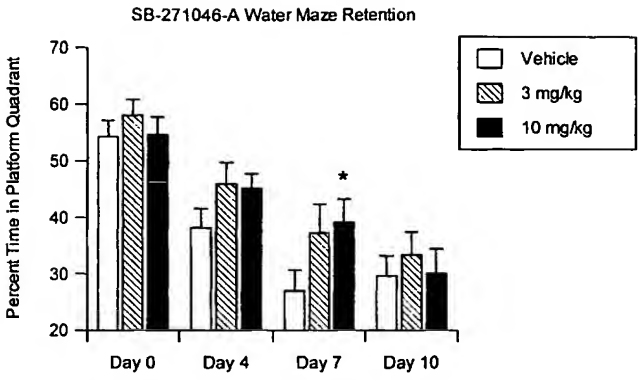

Fig. 2 Effect of SB-271046-A (3 and 10 mg/kg, study 1) on water maze retention. Data are presented as mean±SEM of percentage time spent in the platform quadrant during transfer tests carried out immediately after training (day 0) and at 4, 7 and 10 days after training (n=10). *P<0.05 versus vehicle group

tion phase of this study. There was no significant effect of treatment on acquisition of the platform position as measured by latency [F(2,24)=0.96, P=0.40, Fig. 1] or pathlength [F(2,24)=1.21, P=0.32, data not shown]. There was no significant effect of treatment on the percentage time spent in the platform quadrant during the first transfer test carried out at the end of acquisition [F(2,27)=0.51, P=0.61, Fig. 2 – day 0]. In addition, there was no significant effect of treatment on pathlength (swimming speed) during the first transfer test [F(2,27)=1.47, P=0.25, data not shown]. Retention of the platform position, as assessed by percent time spent in the platform quadrant during subsequent transfer tests, declined when measured 4, 7 and 10 days after training. Repeated measures ANOVA of the transfer test data indicated no overall significant effect of treatment on percentage time spent in the platform quadrant [F(2,27)= 1.74, P=0.19, Fig. 2]. However, examination of the data indicated that SB-271046-A did produce an increase in performance of this retention task at day 4 and day 7 (Fig. 2). ANOVA of the day 7 transfer test data revealed a significant effect of treatment [F(2,27)=3.47, P=0.046], and individual comparisons indicated a significant difference between the vehicle and 10 mg/kg groups (P=0.044). Repeated measures ANOVA indicated that there was no effect of treatment on the number of platform crossings recorded during the transfer tests [F(2,27)=3.10, P=0.097, data not shown].

Following analysis of the data from this experiment, it was observed that there was no further decline in performance in the control group after the day 7 retention test and therefore all subsequent experiments were carried out without the day 10 retention test. In addition, a power calculation determined that a group size of 16 was required to detect a 14% difference between the treatment response and control in the transfer tests, and a second study was carried out to confirm the effects described above. In the second experiment, all rats performed this task well and learnt the position of the platform during the acquisition phase of this study. Again,

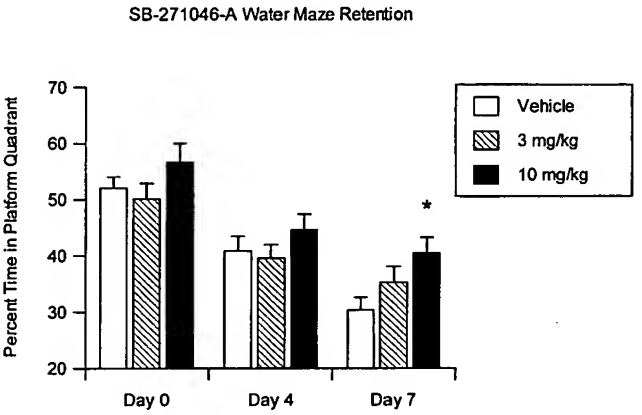

Fig. 3 Effect of SB-271046-A (3 and 10 mg/kg, study 2) on water maze retention. Data are presented as mean±SEM of percentage time spent in the platform quadrant during transfer tests carried out immediately after training (day 0) and at 4 and 7 days after training (n=16). *P<0.05 versus vehicle group

there was no significant effect of treatment on acquisition of the platform position as measured by latency or path length (data not shown). There was also no significant effect of treatment on the percentage time spent in the platform quadrant during the first transfer test carried out at the end of acquisition [F(2,45)=1.43, P=0.25, Fig. 3 – day 0]. Retention of the platform position, as assessed by percent time spent in the platform quadrant during subsequent transfer tests, declined when measured 4 and 7 days after training. Repeated measures ANOVA of the transfer test data indicated a significant effect of treatment on percentage time spent in the platform quadrant [F(2,45)=3.69, P=0.033, Fig. 3]. As seen in the first experiment, ANOVA of the day 7 transfer test data revealed a significant effect of treatment [F(2,45)=3.64, P=0.034], and individual comparisons indicated a significant difference between the vehicle and 10 mg/kg groups (P=0.01). There was no significant effect of treatment on the number of platform crossings re-

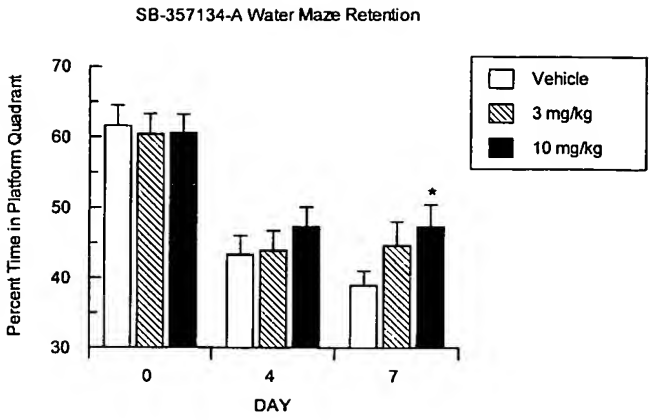

Fig. 4 Effect of SB-357134-A (3 and 10 mg/kg) on water maze retention. Data are presented as mean±SEM of percentage time spent in the platform quadrant during transfer tests carried out immediately after training (day 0) and at 4 and 7 days after training (n=16). *P<0.05 versus vehicle group

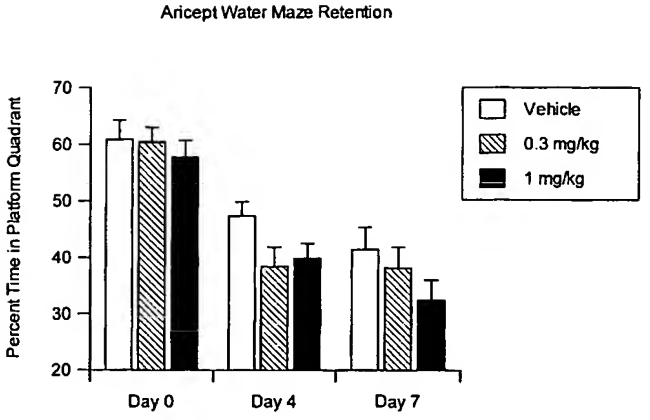

Fig. 5 Effect of Aricept (0.3 and 1 mg/kg) on water maze retention. Data are presented as mean±SEM of percentage time spent in the platform quadrant during transfer tests carried out immediately after training (day 0), and at 4 and 7 days after training (n=16)

corded during the transfer tests [F(2,45)=0.77, P=0.47, data not shown].

SB-357134-A

All rats performed this task well and learnt the position of the platform during the acquisition phase of this study. Again, there was no significant effect of treatment on acquisition of the platform position as measured by latency or path length (data not shown). Repeated measures ANOVA of the transfer test data indicated no significant effect of treatment on percentage time spent in the platform quadrant [F(2,45)=1.39, P=0.26, Fig. 4). In addition, there was no significant effect of treatment on path length (swimming speed) during the first transfer test (data not shown). As seen previously, examination of the data indicated that SB-357134-A did produce an increase in performance of this retention task at day 7 (Fig. 4). ANOVA of the day 7 transfer test data revealed a significant effect of treatment [F(2,45)=3.31, P=0.045], and individual comparisons indicated a significant difference between the vehicle and 10 mg/kg groups (P=0.014). In this experiment, repeated measures ANOVA revealed an overall significant effect of treatment on the number of platform crossings recorded during the transfer tests [F(2,45)=5.40, P=0.008] and individual comparisons indicated a significant increase in the number of platform crosses by the 10 mg/kg group compared to the vehicle group (P=0.015, data not shown).

Aricept

All rats performed this task well and learnt the position of the platform during the acquisition phase of this study. There was no significant effect of treatment on acquisition of the platform position as measured by latency [F(2,45)=1.35, P=0.27] or path length [F(2,45)=2.46, P=0.10], data not shown. There was also no significant effect of treatment on path length (swimming speed) during the first transfer test (data not shown). Repeated measures ANOVA of the transfer test data indicated no significant effect of treatment on percentage time spent in the platform quadrant [F(2,45)=2.70, P=0.08, Fig. 5]. In this experiment, examination of the data indicated that Aricept did not improve the percentage time spent in the platform quadrant. This was confirmed by ANOVA of the day 7 transfer test data which revealed no significant effect of treatment [F(2,45)=0.25, P=0.14]. There was no effect of treatment on the number of platform crossings recorded during the transfer tests [F(2,36)=0.19, P=0.83, data not shown].

Discussion

The aim of the present study was to investigate the effects of the selective 5-HT_6 receptor antagonists SB-271046-A and SB-357134-A, on learning and memory in young, unimpaired rats using a water maze spatial task. These data indicate that systemic administration of both SB-271046-A and SB-357134-A improve some aspects of cognitive performance in this task. There was no significant effect of SB-271046-A or SB-357134-A on acquisition of the hidden platform position in the water maze. However, there was a significant enhancement of retention, which was measured by the percentage time spent in the platform quadrant when tested 7 days after completion of training.

The use of repeated transfer tests reported in the present study is an extension of the normal procedure employed in water maze learning and memory tasks. As such, care has to be taken in the interpretation of these data. The use of three transfer tests during which the rats do not encounter the platform can be regarded as an ex-

tinction procedure. The significant increase in time spent in the platform quadrant on day 7 may not be the consequence of improved retention, but a result of behavioural inflexibility. The rats may in fact fail to redirect their search to other areas of the maze. Although this interpretation cannot be discounted, examination of the swim pattern of individual trials during the transfer tests did not indicate that the vehicle treated animals had redirected their search strategy to other parts of the maze. In addition, as discussed below, there is increasing evidence that 5-HT_6 receptor antagonists are involved in facilitation of cognitive function and to date, there are no reported observations of behavioural inflexibility in other animal models. In the experiments reported here, all training and transfer tests were carried out following administration of test compounds, and further studies are required to determine whether state-dependency played a role in these effects. It will be of interest, for example, to explore whether the enhancement of retention can be facilitated without administration of compound prior to the transfer tests, or on the other hand, whether these effects are replicated if the compound is not administered during training, but only prior to the transfer tests.

There is increasing evidence that 5-HT systems may be involved in the treatment and pathogenesis of cognitive disorders (Meneses 1998) and cognitive dysfunction is associated with ageing and a wide range of neurological and psychiatric disorders. The relationship between 5-HT_6 receptor antagonism and other neurotransmitter systems is at present unclear. Studies with the selective 5-HT_6 receptor antagonist Ro-6790, have supported previous findings with antisense oligonucleotides which suggest that the 5-HT_6 receptor is involved in the control of acetyl-choline neurotransmission in the rat brain (Bourson et al. 1998; Bentley et al. 1999). We have not been able to replicate these effects with SB-271046-A (unpublished data), and further studies are required to determine the extent to which the effects of SB-271046-A and SB-357134-A reported here, are mediated via the cholinergic system. The acetyl-cholinesterase inhibitor Aricept, had no effect on any measure in this study. This is in agreement with previous studies which have reported Aricept to have no effect on water maze learning in unimpaired rats (Van der Staay et al. 1996). Acetylcholinesterase inhibitors have been reported previously to enhance cognition in rat maze tasks, although these effects have been largely restricted to procedures in which deficits have been induced pharmacologically, by administration of scopolamine for example (Braida et al. 1996, Hang Cheng et al. 1996), or by basal forebrain lesions (Ohara et al. 1997).

More recently, excitatory amino acid neurotransmission has also been implicated in the function of 5-HT_6 receptor antagonism and it has been demonstrated that SB-271046-A increases extracellular levels of glutamate and aspartate in the frontal cortex of rats (Dawson et al. 2000). A number of studies are in progress that will increase our understanding of the role of the 5-HT_6 receptor in neurological and psychiatric mechanisms. For example, a recent study (Wooley et al. 2000) demonstrated that Ro-046790 attenuates a scopolamine-induced deficit in an object discrimination task, and provides further support for our observations that selective 5-HT_6 receptor antagonists may facilitate cognitive function in the rat.

In summary, we have demonstrated that systemic administration of SB-271046-A and SB-357134-A produce significant improvements in retention of a spatial memory task in the rat. This enhancement of a previously learned task in a preclinical model may predict an action of SB-271046-A and SB-357134-A upon memory processes in humans.

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A role for 5-ht$_6$ receptors in retention of spatial learning in the Morris water maze

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Abstract

This study investigates the effect of intracerebroventricular administration of a 5-ht$_6$ antisense oligonucleotide (AO) complementary to bases 1–18 of the rat 5-ht$_6$ cDNA initiation sequence (Mol. Pharmacol. 43 (1993) 320) (1.5 μg twice daily for six days) and i.p. injection of a selective 5-ht$_6$ receptor antagonist Ro 04-6790 (10 or 30 mg/kg once daily for three days) on acquisition and retention in the Morris water maze. Neither the 5-ht$_6$ AO (which reduced cortical [^{3}H]-LSD binding sites by 10–16%) nor Ro 04-6790 affected acquisition, but both enhanced retention of the learned platform position such that rats spent significantly longer searching the trained platform position than any other area during the probe tests. Furthermore, neither AO nor Ro 04-6790 had any effect on the time taken to reach a raised visible platform, indicating that visual acuity was unimpaired. In addition, AO reduced both food consumption and body weight and the later effect was also seen following Ro 04-6790, suggesting a role for the 5-ht$_6$ receptor in the regulation of feeding. Hence, while the underlying mechanism remains unclear, enhanced retention of spatial learning following both AO and 5-ht$_6$ antagonist administration strongly indicate a role for this receptor in memory processes.

Keywords: 5-ht$_6$ Receptor; Antisense oligonucleotide; Ro 04-6790; Morris water maze; Spatial learning; Hippocampus

1. Introduction

Mammalian 5-HT receptors (14) have been cloned and sequenced (Barnes and Sharp, 1999). One of the most recent additions is the 5-ht$_6$ receptor that was defined by molecular biology rather than pharmacological tools (Branchek and Blackburn, 2000). Both the rat and human (Ruat et al., 1993; Monsma et al., 1993; Kohen et al., 1996) 5-ht$_6$ receptor have been cloned and hydropathic analysis demonstrates that it has a typical seven transmembrane spanning G-protein linked structure and is positively coupled to adenylyl cyclase. The 5-ht$_6$ receptor mRNA is highly expressed in the limbic and cortical regions of the rat brain, including the olfactory tubercles, striatum, nucleus accumbens and hippocampus (Monsma et al., 1993; Ruat et al., 1993; Ward et al., 1995; Kohen et al., 1996; Gérard et al. 1996, 1997). The general consensus is that the 5-ht$_6$ receptor is not expressed in the periphery, although one group did report 5-ht$_6$ mRNA in the stomach of the guinea pig (Ruat et al., 1993). By using polyclonal antibodies directed against the C-terminal region of the 5-ht$_6$ receptor, Gérard et al. (1997) identified diffuse immunoreactivity in the neuropil of structures such as the olfactory tubercles, dendrites in the granular cell layer in the hippocampus and the medium spiny neurones in the caudate putamen of the striatum.

Prior to the recent development of selective, 5-ht$_6$ receptor antagonists (Sleight et al., 1998; Bromidge et al., 1999; Isaac et al., 2000) tools for the in vivo characterisation of 5-ht$_6$ receptor function were limited. Chronic intracerebroventricular (i.c.v.) injection of a 5-ht$_6$ receptor specific antisense oligonucleotide (AO) directed against the initiation codon region of the mRNA has been successfully used by several groups to down-regulate 5-ht$_6$ receptor expression in the CNS and deter-

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mine concomitant behavioural alterations (Bourson et al., 1995; Yoshioka et al., 1998; Hamon et al., 1999). In the initial study (Bourson et al., 1995), chronic i.c.v. injection of a 5-ht$_6$ receptor-directed AO was found to elicit a specific behavioural syndrome comprised of yawning, chewing and stretching which was accompanied by a 30% decrease in [^{3}H]-LSD binding compared with that in controls, providing the first evidence that the 5-ht$_6$ receptor was functionally expressed in the rat brain. With a similar AO protocol, Yoshioka et al. (1998) reported attenuation of conditioned fear stress-induced 5-HT release using cortical microdialysis probes in conscious rats, suggesting that the 5-ht$_6$ receptor may be involved in certain anxiety disorders. In support of this suggestion, Hamon et al. (1999), recently reported that a similar AO treatment decreased social interaction and the percentage time spent on the open arms of an elevated plus maze.

Recently a number of selective 5-ht$_6$ antagonists have been characterised (Sleight et al., 1998; Bromidge et al., 1999; Isaac et al., 2000) enabling the specificity of 5-ht$_6$ receptor antisense-induced behavioural effects to be determined. The first centrally active 5-ht$_6$ receptor antagonist to be developed, 4-amino-*N*-(2,6-bis-methylamino-pyramidin-4-yl)-benzene sulphonamide (Ro 04-6790), has a pK_i of 7.35 and 7.26 for the rat and human 5-ht$_6$ receptor, respectively, and over 100-fold selectivity for this compared with 24 other binding sites (Sleight et al., 1998). Initial behavioural studies showed that Ro 04-6790, produced a dose-related yawning and stretching syndrome analogous to that observed with 5-ht$_6$ receptor AO treatment (Sleight et al., 1998). Furthermore, the stretching behaviour induced by both the 5-ht$_6$ receptor AO and Ro 04-6790 were attenuated with non-selective muscarinic antagonists (atropine (both studies) and scopolamine (Ro 04-6790 only)), but not by the dopamine antagonist, haloperidol. This data suggested that there was a cholinergic, but no dopaminergic, component to this behaviour (Bourson et al., 1995; Bentley et al., 1999). Although a second selective 5-ht$_6$ receptor antagonist, SB-271046 did not induce yawning when administered alone, it enhanced physostigmine-induced yawning (Routledge et al., 1999) in agreement with the role of the 5-ht$_6$ receptor in this behaviour. The high level of expression of the 5-ht$_6$ receptor in the hippocampus (Ruat et al., 1993; Bourson et al., 1995; Ward et al., 1995; Gérard et al., 1997) together with evidence for a link with cholinergic function suggests a role for this receptor in memory processes. Two preliminary studies have suggested that both the 5-ht$_6$ receptor AO (Bentley et al., 1997) and the 5-ht$_6$ receptor antagonist SB-271046 (Rogers et al., 1999) can enhance retention, but not acquisition, of a spatial learning task in the rat. This study provides the first detailed account of the role of the 5-ht$_6$ receptor in a memory paradigm by comparing the effect of administration of the 5-ht$_6$ receptor-directed AO with that of a selective 5-ht$_6$ antagonist on performance in the Morris water maze.

2. Methods

2.1. Animals

Adult male, Lister hooded rats (Biomedical Services Unit, University of Nottingham derived from Charles River stock) weighing 280–310 g at the beginning of the study (group A, Fig. 1), were singly housed on a 12 h light–dark cycle (lights on at 07.00 hours) and given a weighed amount of food and water ad libitum. Room temperature (21±1°C) and humidity (55–65%) were maintained constant. A second group (group B, Fig. 1) of male, Lister hooded rats (Charles River, UK weighing between 250 and 260 g at the start of the study were brought in-house in groups of four under the conditions described above one week prior to the commencing experimentation. All experiments were performed in accordance with the UK Animals (Scientific Procedures) Act 1986 using a blind protocol.

2.2. Oligodeoxynucletides (ODNs)

5-ht$_6$ receptor-directed antisense (AO) and scrambled (SO) oligodeoxynucleotides (ODNs) were similar to those used by Bourson et al. (1995). The AO being complementary to bases 1–18 of the initiation codon region (5′-GCC TGG CTC TGG AAC CAT-3′) of the rat 5-ht$_6$ receptor cDNA (Monsma et al., 1993). The control sequences employed were a SO (identical to that used by Bourson et al. (1995), consisting of a randomised order of the AO bases (5′-CGC TCA GTC ATC GGA GTC-3′), and, in addition, a mismatch (MO, 5′-

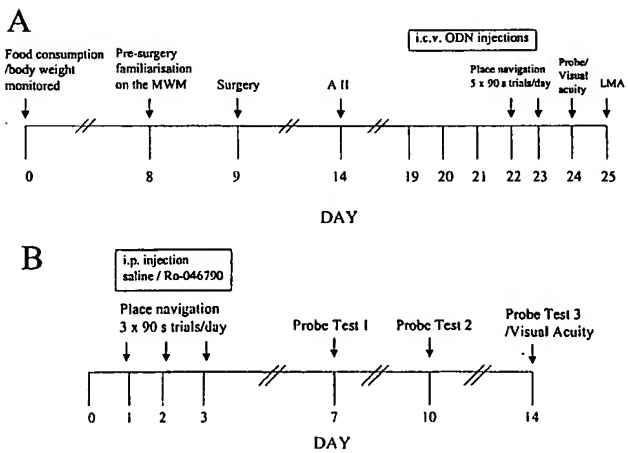

Fig. 1. Protocol for the ODN (group A) and Ro 04-6790 (group B) experiments on place navigation training and food intake. MWM — Morris water maze; AII — angiotensin II (200 ng i.c.v. in 0.154 M saline); LMA — locomotor activity.

GCA TGG CGC TGT AAC CCT-3′) sequence consisting of four base pair changes compared with the AO. All ODNs were end-capped phosphorothioate derivatives (only the terminal two bases being modified as used by Hamon et al. (1999)) to reduce non-specific toxicity (Hebb and Robertson, 1997). In an attempt to further reduce possible side-effects, a lower dose of AO than reported previously was used (1.5 μg/day compared with 12–24 μg/day, Bourson et al., 1995). Each ODN (0.75 μg/μl) was dissolved in sterile saline (0.154 M) that was used as the vehicle control.

2.2.1. Intracerebroventricular (i.c.v.) injection of ODNs

A 23G stainless steel cannula was implanted under halothane (2–4% nitrous oxide) anaesthesia above the left lateral ventricle, (0.9 mm posterior to Bregma, 1.4 mm lateral to the midline and to a depth of 1.8 mm, Paxinos and Watson, 1986) 10 days prior to i.c.v. administration of ODNs using a Kopf stereotaxic frame and dental cement. Subsequent i.c.v. injections were performed using a 3.8 mm long 31G injection needle which penetrated into the lateral ventricle. The guide cannula was kept patent by keeping a steel stylet in the guide cannula between injections. Five days prior to the administration of the ODNs, the cannula position was confirmed using i.c.v. injection of Angiotensin II (200 ng/μl, AII), which in all cases elicited a dipsogenic response within 2–3 min of administration on returning the rat to the home cage (Fig. 1A).

2.3. Drug or ODN treatment

Ten days after surgery, animals in group A received one of the four treatments (saline, 5-ht$_6$-directed AO, mismatch oligonucleotide (MO) and a scrambled oligonucleotide sequence (SO) twice daily (between 08.30–10.00 hours and 16.00–17.30 hours) for six days (Fig. 1A). ODNs were injected rapidly in a volume of 1 μl, and the injection needle was left in place for an additional 60 s to ensure dispersal of injectate into the cerebrospinal fluid. A second group of rats (Fig. 1B), received saline or Ro 04-6790 (10 or 30 mg/kg i.p.) 30 min prior to training for three rather than two consecutive days in the Morris water maze.

2.4. Behavioural parameters

2.4.1. The Morris water maze

A 2 m diameter, 0.7 m high fibreglass circular water maze filled with water (21°C) made opaque by the addition of an industrial opacifier (500 ml, Taski Calcacio 7244048, F Hoffmann-La Roche) was positioned underneath a wide-angled video camera to track animal activity (CPL Systems, Cambridge, UK). The 10 cm^2 perspex platform, lying 1.5 cm below the water surface, was placed in the centre of one of the four imaginary quadrants which remained constant for each rat. Prominent visual cues (black patterns on white card and a patterned hospital curtain) surrounding the maze were used as spatial cues around the arena (200 lux light intensity).

Rats receiving ODN underwent a thigmotaxis trial (120 s free swim with no platform present) one day prior to surgery in order to introduce them to the maze arena. On days 4 and 5 of ODN/saline treatment, rats received 5×90 s trials (Fig. 1A). The maze training protocol was slightly modified for the 5-ht$_6$ antagonist study to ascertain whether antagonism of the 5-ht$_6$ receptor might be associated with an impairment of retention during the acquisition period (as suggested from the antisense experiment) and to enable results to be compared with those reported in a preliminary study using a structurally unrelated 5-ht$_6$ receptor antagonist (Rogers et al., 1999). The second group of rats received 3×90 s trials on each of the three days of 5-ht$_6$ receptor antagonist treatment (Fig. 1B). Thus, the rats in both groups A and B received a very similar total number of trials in the maze. On each trial, the rat was released into the maze facing the side wall starting from a different position (the adjacent clockwise one) in one of the three quadrants not containing the platform. The trial ended when either 90 s had elapsed or the rat climbed onto the platform, where it was allowed to remain for 20 s before being removed from the arena for a 30 s inter-trial period. If the rat did not locate the platform it was guided to it, and left on it for 20 s before removal from the arena for 30 s. The parameters measured were latency to reach the hidden platform (s), distance travelled (m) and swim speed (m/s). On day 6 of ODN injection (group A) or on days 4, 7 and 11 following Ro 04-6790 or saline treatment (group B) a probe test (60 s free swim without a platform) was performed and the time (s) spent in a 10 cm annulus around each possible platform position and the swim speed (m/s) were recorded. In both groups after the last probe test a visual acuity test was performed. The time taken to climb onto a raised visible platform (with black tape affixed around the rim) placed in the quadrant opposite to the training position was recorded to assess any visual impairment.

2.4.2. Animal activity monitor

Sixteen hours after the final ODN injection, each rat was placed into a perspex computerised activity monitor box (23.6 cm×38.6 cm×30 cm) surrounded by three layers of infrared beams to monitor gross locomotor activity and rearing (Clemett et al., 1998). Activity data was analysed as the total number of beam breaks in three consecutive 20 min periods.

2.4.3. Food consumption and body weight

In animals receiving ODN treatment food intake was monitored daily at 09.00 hours (by weighing the amount of food not consumed during the previous 24 h) and

body weight measured twice weekly until the first i.c.v. injection and daily thereafter (Fig. 1A). Rats receiving the 5-ht$_6$ receptor antagonist were weighed daily at 09.00 hours for the duration of the experiment (Fig. 1B).

2.4.4. Statistical analysis

Data was analysed using a two factor ANOVA, followed where necessary, by a one factor ANOVA and Duncan's New Multiple Range post hoc test; $p < 0.05$ was considered significant.

2.5. Membrane preparation and [^{3}H]-LSD binding

Following the last behavioural test, the rats were killed, their brains dissected out, weighed, snap frozen in liquid nitrogen and stored at −80°C until required. Membrane preparation and [^{3}H]-LSD binding was performed according to the method of Bourson et al. (1995) in the presence of 300 nM spiperone to prevent binding to 5-HT$_{1A}$ and dopamine D$_2$ receptors. In brief, eight-point saturation assays were performed three times on homogenates prepared from the anterior 3 mm of the cortex that had been pooled according to rat treatment. B_{max} and K_d values were generated using GraphPad Prism software. Since all tissue from each group of animals was pooled into a single preparation, it was not appropriate to perform statistical analysis on the resultant group data.

3. Results

3.1. Acquisition

3.1.1. Antisense administration

All rats receiving ODNs or saline progressively learned to locate the hidden platform ($p < 0.05$, by trial) during the 10 successive trials in the water maze (Fig. 2A) such that there was no significant difference irrespective of the treatment received. On the first day of training (trials 1–5), there was no drug-induced difference in the ability of the rats to locate the hidden platform (ANOVA, $F_{(3,95)} = 0.60$; $p = 0.62$, by treatment). However, on the second day (trials 6–10) the overall average escape latency for all five trials in AO-treated rats (18 s) was lower than that of SO (38 s), MO (20 s) and saline (29 s) groups (ANOVA, $F_{(3,95)} = 3.76$; $p = 0.013$ by treatment). While not significant, the data shows that this was primarily due to a shorter latency to reach the platform on the first (sixth) trial on the second day of testing in the AO group compared with that of other groups. The distance swum (Fig. 2B) during each successive trial showed a profile similar to the latency to reach the hidden platform. However, the average swim speed (Fig. 2C) was equivalent in all groups on all trials irrespective of the treatment received.

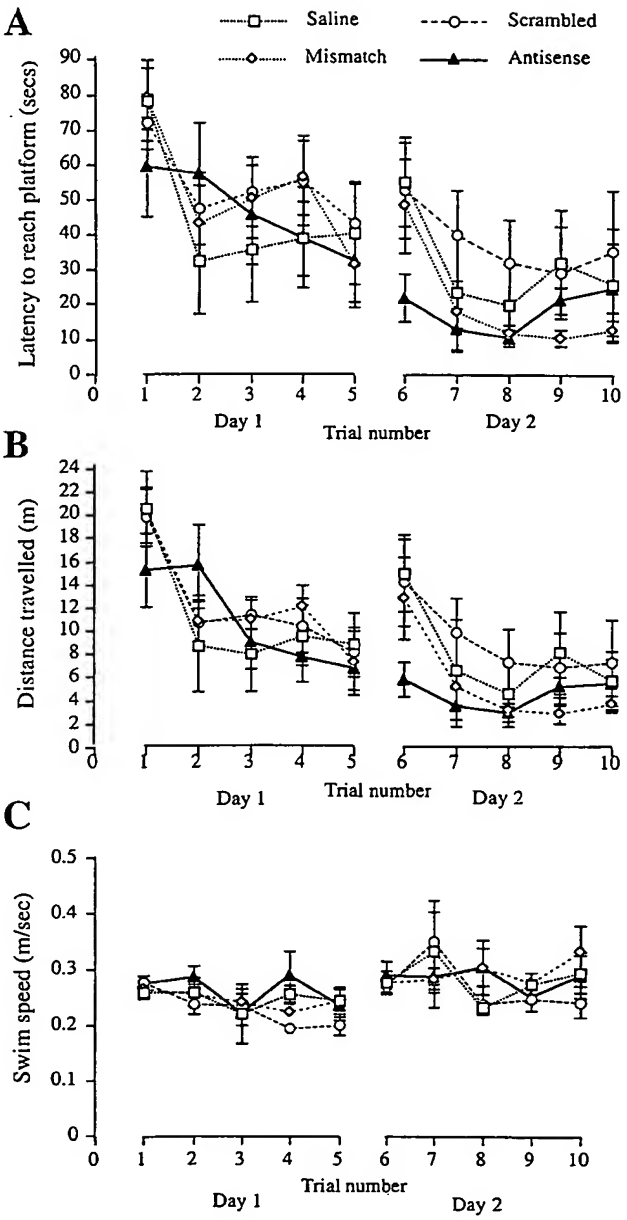

Fig. 2. (A) Latency (s, mean±SEM) and (B) distance travelled (m, mean±SEM) by ODN/saline treated rats to find a hidden platform (5 trials/day for two days) in the Morris water maze. In both cases, all four groups progressively learned to locate the hidden platform (ANOVA, $F_{(3,190)}$=7.97; p=0.0001 by trial (1–10)). (C) Swim speed (m/s, mean±SEM) throughout the training process was equivalent on all trials irrespective of treatment group.

3.1.2. Antagonist administration

Overall, in the water maze rats given Ro 04-6790 displayed a similar learning profile to rats treated with AO. All groups progressively learnt to locate the hidden platform with successive trials (Fig. 3A) irrespective of drug treatment. On the first two days of training (trials 1–6) Ro 04–6790 had no effect on the ability of the rats to locate the hidden platform. In contrast, on the third day (trials 7–9) the overall escape latency for all three trials

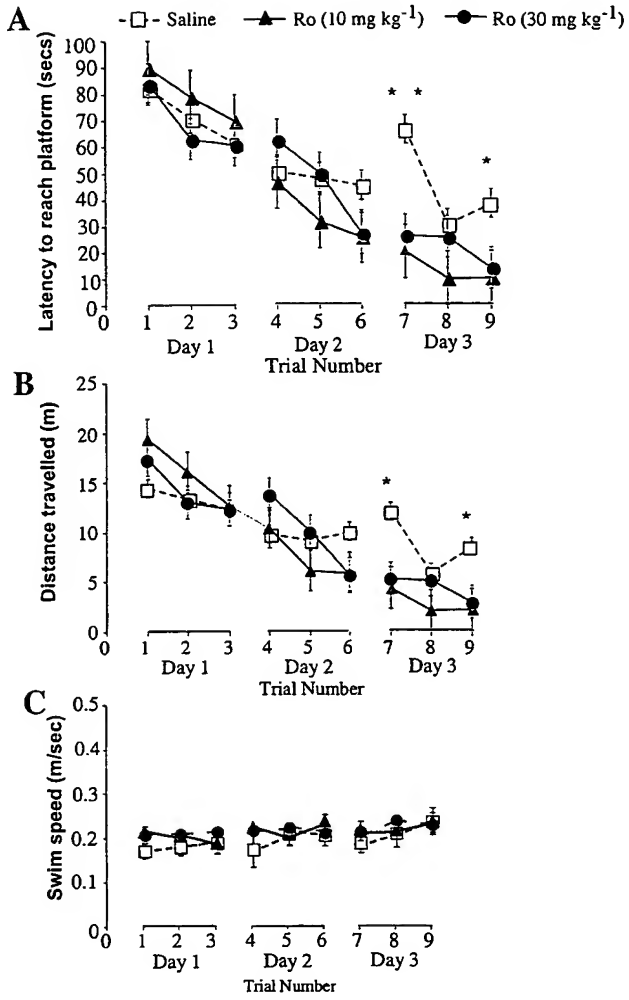

Fig. 3. (A) Latency (s, mean ± SEM) and (B) distance travelled (m, mean±SEM) by Ro 04-6790/saline treated rats to find a hidden platform (3 trials/day for three days in Morris water maze). All three groups progressively learnt to locate the hidden platform (ANOVA, $F_{(8,\ 188)} = 15.0$; $p = 0.0001$, by trial (1–9)). (C) Swim speed (m/s, mean±SEM) throughout the training process was equivalent on all trials irrespective of treatment group.

in rats treated with both 10 mg/kg Ro 04-6790 (14 s) and 30 mg/kg Ro 04-6790 (22 s) was significantly lower than that of saline-treated rats (45 s). Further analysis revealed a significant reduction of the overall escape latency in the seventh and ninth trials in rats treated with Ro 04-6790 (ANOVA, $F_{(2,21)} = 4.379$; $p = 0.026$, and $F_{(2,21)} = 6.46$; $p = 0.007$, by treatment; trials 7 and 9, respectively) although the escape latency for saline-treated animals in these trials was unexpectedly elevated when compared to that in the preceding trials. The total distance swum (Fig. 3B) by the rats showed a similar profile to the latency to reach the hidden platform. Ro 04-6790 had no effect on swim speed with the average swim speeds equivalent in all groups (Fig. 3C).

3.2. Retention

3.2.1. Antisense administration

With the hidden platform removed, each rat was placed into the maze for 1 min and the time spent swimming in the 10 cm annuli surrounding the four theoretical platform positions was measured (Fig. 4). Rats receiving saline and MO spent an equal amount of time in each 10 cm annuli, while SO-treated rats spent significantly longer ($p < 0.05$) searching the correct training platform position compared with that in the quadrant to the right of it. Furthermore, AO-treated animals showed a completely different search profile from those in the other three treatment groups, spending significantly longer ($p < 0.05$) searching the correct annulus surrounding the training platform position than the other three platform areas (Fig. 4A). The total distance swum and average swim speed of all four ODN treatment groups were equivalent during the probe test.

3.2.2. Antagonist administration

In the first probe test, four days following the last training trial, neither dose of the selective 5-ht$_6$ receptor antagonist had any effect on the time spent exploring the correct training platform position. All rats spent significantly longer exploring the correct platform position than any of the other three areas examined (Fig. 4B). In contrast, in the second probe test performed seven days following the last training trial, rats treated with saline and 10 mg/kg Ro 04-6790 spent a roughly equal time exploring each platform annuli, while rats given the highest dose of Ro 04-6790 spent significantly longer exploring the training platform position than any other annuli (ANOVA, $F_{(3,\ 28)} = 3.9$; $p = 0.02$) (Fig. 4C). A similar profile was observed in the third probe test, performed 11 days following the cessation of training in the same rats such that rats pre-treated with saline and 10 mg/kg Ro 04-6790 spent an equal time exploring each platform annuli. By comparison, rats pre-treated with 30 mg/kg Ro 04-6790 spent significantly more time exploring the correct annulus than the one on the left or on the right ($p < 0.05$) and also appeared to spend less time in the opposite annulus, but this just failed to reach significance ($p = 0.08$) (Fig. 4D). In all the three probe tests, both the total distance swum and swim speed were equivalent in all the three treatment groups.

3.3. Visual acuity

Treatment with neither ODN (i.c.v.) nor the selective 5-ht$_6$ receptor antagonist Ro 04-6790 (i.p.) had any effect on the time taken to reach the raised visible platform, indicating no long-term effect of any treatment on visual acuity.

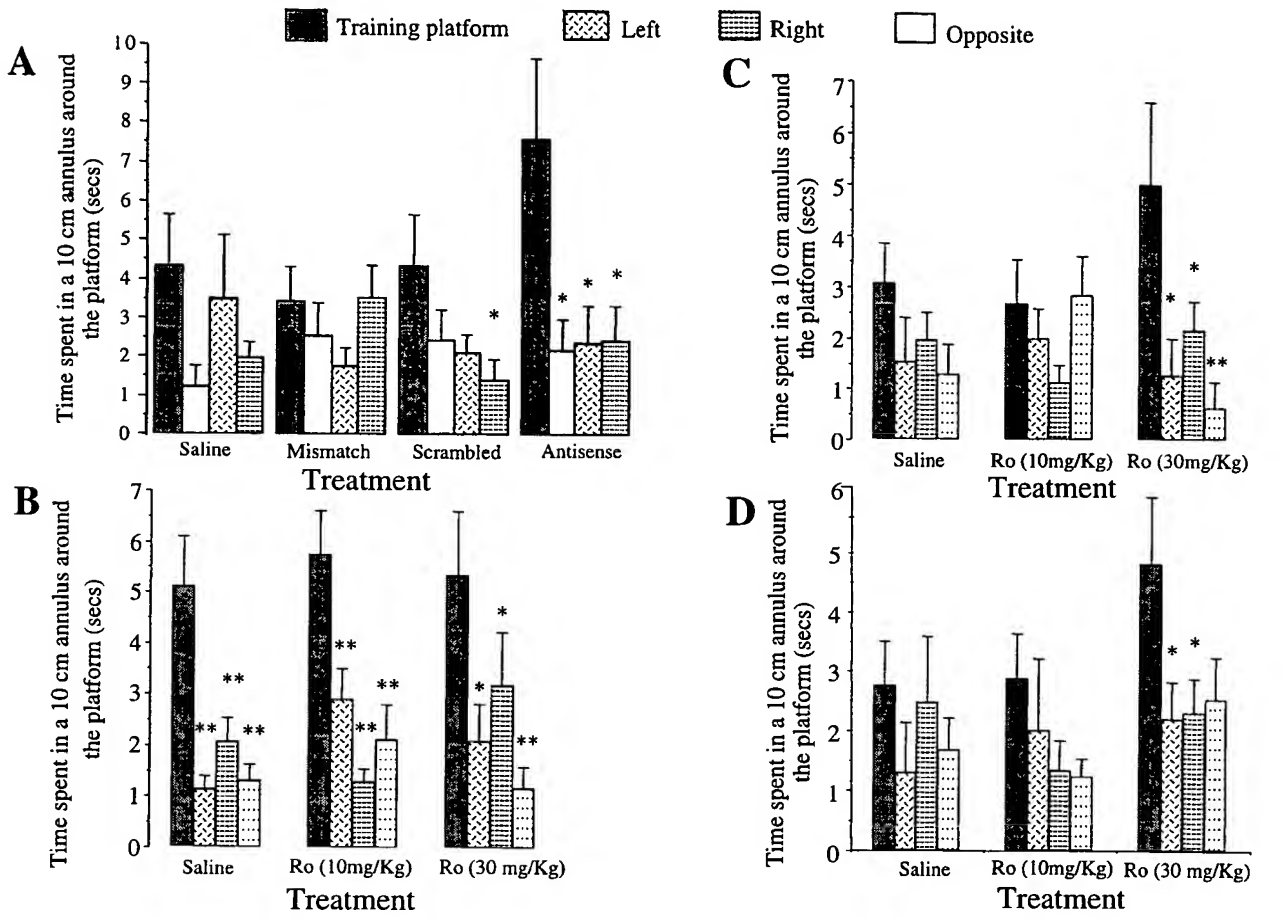

Fig. 4. Probe tests to verify that each rat had learned the position of the hidden platform and to investigate changes in retention of the task. The amount of time spent in a 10 cm annulus surrounding each theoretical platform position (s, mean±SEM) was determined in saline, MO-, SO- and AO-treated rats (A) and in rats pre-treated with saline or Ro 04-6790; 4 days (B), 7 days (C) and 11 days (D) after the last acquisition trial. *$p < 0.05$; ** $p < 0.01$; ***$p < 0.001$ compared with the time spent in the platform position used for training that rat (Duncan's New Multiple range following one way ANOVA).

3.4. Food consumption and body weight

Daily food consumption was comparable in all animals prior to surgery and i.c.v. ODN/saline injection (Fig. 5A) and was transiently reduced by both surgery and angiotensin II injection in all treatment groups. Twenty-four hours after the first injection, rats receiving AO ate less than MO-treated rats on the second day of injection ($p < 0.05$) and less than saline controls on the third day of injection ($p < 0.05$).

The body weight of rats in all four ODN and saline groups steadily increased prior to surgery and i.c.v. injection (Fig. 5B). Initially, there was no significant change in body weight irrespective of the i.c.v. drug injected. However, as expected from the reduction in food consumption seen on the 3rd, 6th and final (ODN free) day of injection, AO-treated rats had a significantly lower ($p < 0.05$) body weight than SO-treated animals.

Fig. 5C shows that in the antagonist study body weight was equivalent prior to treatment with saline or Ro 04-6790. The highest dose of Ro 04-6790 (30 mg/kg) prevented weight gain from the morning after the first to the morning after the last injection when compared with rats given saline or 10 mg/kg Ro 04-6790; such that these rats had a lower body weight thereafter (ANOVA, $F_{(2,273)} = 14.381$; $p = 0.0001$ by treatment). Following the last injection, even in rats given the highest dose of Ro 04-6790, the daily body weight gain was similar to that in rats given saline or 10 mg/kg Ro 04-6790. However, rats given the highest dose of Ro 04-6790 retained a lower overall body weight throughout the remainder of the experiment and failed to compensate for their weight loss during the three treatment days.

3.5. Locomotor activity

Chronic administration of the 5-ht$_6$ receptor-directed AO had no effect on locomotor activity (including head-dipping and rears) in computerised activity boxes com-

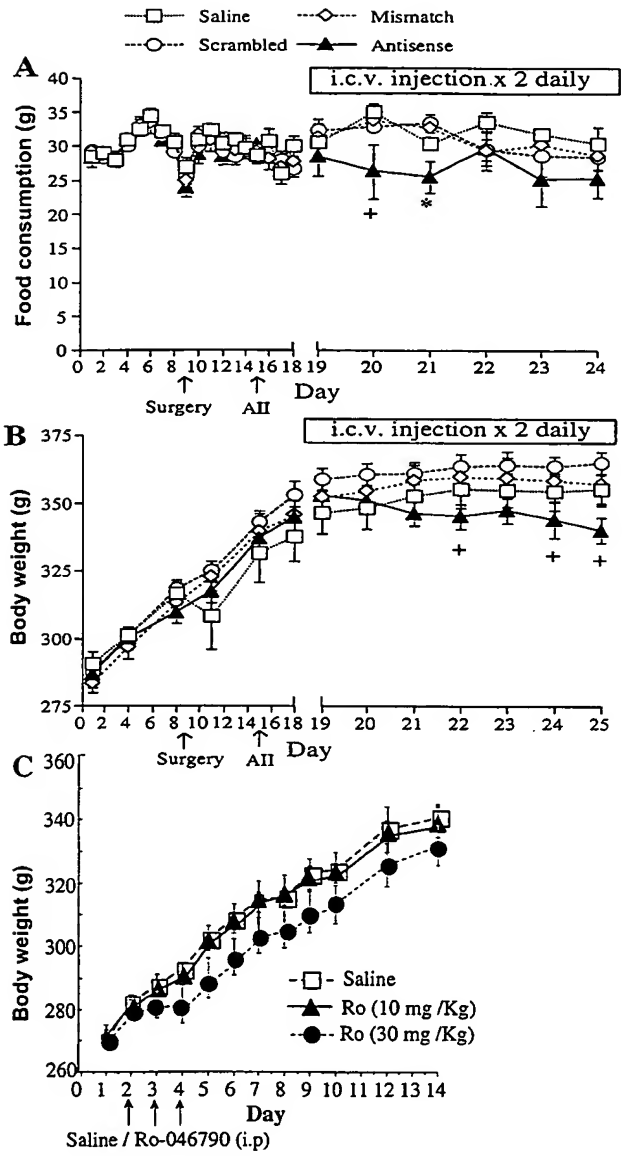

Fig. 5. (A) Daily food consumption and (B) body weight (g, mean±SEM) of rats before, during and after chronic i.c.v. injection of saline (2 ml/day) or 5-ht$_6$ receptor-directed AO, MO, or SO (1.5 g/day). Surgery was performed on day 9 and i.c.v. injections began on day 19, being given twice daily for six days. (A) *$p < 0.05$ compared with saline, +$p < 0.05$ compared with MO controls and (B) +$p < 0.05$ compared with SO controls, Duncan's New Multiple range following one-way and two-way ANOVA from and including day 19; $F_{(3,120)} = 7.80$; $p = 0.0001$ (food intake) and $F_{(3,120)} = 8.72$; $p = 0.0001$(body weight) by treatment. (C) Body weight (g, mean±SEM) of rats during and following withdrawal of treatment with saline or Ro 04-6790 (10 or 30 mg/kg i.p.) as indicated (two-way ANOVA, $F_{(2, 273)} = 14.381$; $p = 0.0001$, by treatment).

pared with the respective ODN and saline controls (data not shown).

3.6. [^{3}H]-LSD binding

In agreement with the previously published data (Bourson et al., 1995; Yoshioka et al., 1998; Matsumoto et al., 1999), AO treatment appeared to reduce the number of cortical [^{3}H]-LSD binding sites by 10–16% when compared with control groups (B_{max} values being 25.3±4.7, 23.5±2.9, and 21.2±4.7 fmol/mg tissue for MO-, SO- and AO-treated animals, respectively, mean±SEM, $n = 1$ each measured three times).

4. Discussion

The results demonstrate that either chronic administration of the 5-ht$_6$ receptor-directed AO or a selective 5-ht$_6$ receptor antagonist during the acquisition period in a spatial learning paradigm does not affect the learning process per se, but may improve long-term memory retention of the task.

The ability to demonstrate a reduction in 5-ht$_6$ receptor levels following treatment with AO is hampered by the lack of selective high affinity ligands for the receptor although the first of such ligands has just been reported (Hirst et al., 2000). Since no selective 5-ht$_6$ receptor radioligand was available when the current experiments were performed, [^{3}H]-LSD was used, as in the previous experiments with this 5-ht$_6$ receptor-directed AO sequence. In the presence of 300 nM spiperone to mask 5-HT$_{2A}$ and dopamine-D$_2$ receptor receptors [^{3}H]-LSD labels a small pool of receptors including the 5-ht$_6$ receptor (Bourson et al., 1995). Although it was not possible to demonstrate a significant reduction in [^{3}H]-LSD binding with the current AO treatment, the apparent reduction in 5-ht$_6$ receptor number seen was similar in magnitude (10–16% from controls) to that reported previously with higher doses of the same AO sequence. Bourson et al. (1995, 12 μg/day×4 days) found a 30% decrease in the cortical [^{3}H]-LSD binding, while Yoshioka et al. (1998, 14 μg/day×7 days) Matsumoto et al. (1999, 12 μg/day×4 days) reported a similar magnitude decrease in whole brain homogenates. Furthermore, Hamon et al. (1999, 2.2 μg/day×4 days of 5-ht$_6$ AO) recently reported an analogous decrease in 5-ht$_6$-like immunoreactive staining in the nucleus accumbens using a polyclonal antiserum. As the level of 5-ht$_6$ binding herein was determined two days after the last AO injection in cortical tissue, the actual change in receptor expression in brain regions closer to the ventricles at the time of the behavioural measurements could be more extensive. In support of the proposal that the behavioural changes elicited by treatment with AO are caused by the down-regulation of the 5-ht$_6$ receptor, the selective 5-ht$_6$ receptor antagonist, Ro 04-6790 produced comparable changes in body weight and retention after a spatial learning paradigm. One way to confirm that the behav-

ioural changes elicited by the current AO and antagonist treatments are both mediated by attenuation of 5-ht_6 receptor function would be to demonstrate that the 5-ht_6 antagonist is ineffective following AO administration. However, a full dose–response analysis with the AO would be required to ensure that a maximal response had been achieved, otherwise an additive effect of such a drug combination could still be observed.

Irrespective of treatment, all rats learnt the location of the hidden platform following repeated exposure to the Morris water maze. Treatment with 5-ht_6 antisense or Ro 04-6790 had little effect on the overall acquisition but suggested an improved performance on the first trial of the second day (trial 6) or trials 7 and 9 of the third trial day, respectively. More notably, treatment with 5-ht_6 antisense or 30 mg/kg Ro 04-6790 enhanced the retention of the learned platform position such that rats spent significantly longer searching the trained platform position than any of the other areas in subsequent probe tests.

While the mechanism for the apparent no-tropic effect of the 5-ht_6 receptor remains unclear, there is a strong indication of a link between the 5-ht_6 receptor and acetylcholine (Bourson et al. 1995, 1998; Bentley et al., 1999; Routledge et al., 1999). Acetylcholine has been implicated in memory function largely because of the degradation and loss of cholinergic markers in the neurodegenerative Alzheimer's disease (Bartus et al., 1982; Bierer et al., 1995). Memory appears to be facilitated by administration of 5-HT receptor antagonists and inhibited following 5-HT receptor activation. For instance, 5-HT_{1A} receptor agonists impair spatial learning (Carli and Samanin, 1992), while 5-HT_{1A} antagonists prevent the impairment of choice accuracy caused by intrahippocampal injections of scopolamine (Carli et al., 1997). Since 5-ht_6 receptors are localised on the distal dendrites of pyramidal and granular cells in the hippocampus (Gérard et al., 1997) and on pyramidal neurones expressing 5-HT_{1A} receptors (Yau et al., 1997), it is possible that 5-ht_6 and 5-HT_{1A} receptors could function in a similar manner to alter mnemonic processes. However, extensive degeneration of serotonergic neurones in the anterior raphé area following microinfusion with 5,7-DHT produced no change in 5-ht_6 mRNA levels in the nucleus accumbens, striatum or hippocampus. This suggests that 5-ht_6 mRNA is not located in serotonergic neurones and that 5-ht_6 receptors are only located post-synaptic to serotonergic neurones and do not function as terminal autoreceptors in the hippocampus (Gérard et al., 1996).

5-ht_6 receptor regulation of cholinergic neurotransmission was proposed by Bourson et al. (1995). This group demonstrated that treatment with a 5-ht_6-directed AO produced a characteristic behaviour comprised of yawning, stretching and chewing which was antagonised by atropine or scopolamine but not by haloperidol. Moreover, it was noted that since the behaviour was only seen after treatment with the AO the 5-ht_6 receptor must either be under the tonic influence of 5-HT or be constituitively active for AO treatment to inhibit cholinergic transmission. This is consistent with data (not shown) that treatment with 5-ht_6 AO has no effect on 5-HT/5-HIAA ratio, which is an index of 5-HT turnover in various brain areas.

More recently Bourson et al. (1998) provided evidence for a regulatory role of 5-ht_6 receptors on cholinergic but not dopaminergic transmission in the striatum. Rats received 6-OHDA to produce unilateral lesions in the medial forebrain bundle. Ro 04-6790 inhibited atropine- and scopolamine-induced ipsilateral rotations but not dopamine-induced contralateral rotations. While the mechanism is unknown, an increase in acetylcholine release following 5-ht_6 receptor blockade is possible (Bourson et al., 1998). In addition, Gérard et al. (1997) suggested that 5-ht_6 receptors were located on GABA containing spiny neurones in the striatum and thus interactions between 5-ht_6 and muscarinic receptors may involve GABAergic neurotransmission. This theory is consistent with immunohistochemical experiments in which 5-ht_6 receptors have been localised onto GABAergic/peptidergic striatopallidal and striatal nigro output pathways (Ward and Dorsa, 1996). The lack of any overt change in locomotion in a novel open field arena (Otano et al., 1999) or in the average swim speed in the current water maze study shows that there is no evidence for sedation or gross motor impairment following 5-ht_6 receptor blockade.

Recent preliminary data with a structurally unrelated, selective, 5-ht_6 receptor antagonist (SB-271046) has provided further evidence for a role of the 5-ht_6 receptor in memory function. In two different models of learning and memory, (operant delayed alternation in aged animals and the water maze, respectively), cognitive enhancement was seen following 5-ht_6 receptor antagonism (Rogers et al., 1999).

During the course of the present ODN experiment, only animals receiving 5-ht_6-directed AO, and not MO or SO, treatment showed a decrease in both food consumption and body weight suggesting that this receptor may also regulate feeding. This data is in contrast with the studies of Yoshioka et al. (1998) and Hamon et al. (1999), in which treatment with the 5-ht_6-directed AO did not alter body weight. However, the reduction in body weight seen with the highest dose of the selective 5-ht_6 receptor antagonist Ro 04-6790 (30 mg/kg) is consistent with a role for the 5-ht_6 receptor in the regulation of feeding. While it has previously been established that 5-HT and several of its receptors play an important role in the process of satiety (Blundell, 1977; Dourish, 1995 for review) this study implicates a novel 5-HT receptor, the 5-ht_6 receptor, in the regulation of feeding.

In summary, this study identifies a role for 5-ht_6 recep-

tors in memory showing enhanced retention of a learnt task in the Morris water maze and in addition suggests it may be involved in modulating feeding.

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Brief Articles

Influence of the 5-HT$_6$ Receptor on Acetylcholine Release in the Cortex: Pharmacological Characterization of 4-(2-Bromo-6-pyrrolidin-1-ylpyridine-4-sulfonyl)phenylamine, a Potent and Selective 5-HT$_6$ Receptor Antagonist†

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A small series of aryl pyridyl sulfones has been prepared and investigated for its 5-HT$_6$ receptor binding properties. Thereof, pyrrolidinyl derivative **11** proved to be a very potent (pK_i 9) and selective 5-HT$_6$ receptor antagonist. By means of in vivo microdialysis in the frontal cortex and a passive avoidance paradigm, where **11** reversed a scopolamine induced retention deficit, a functional correlation between 5-HT$_6$ receptors and cholinergic neurotransmission could be shown, supporting the therapeutic potential of 5-HT$_6$ receptors in the treatment of cognitive deficits.

The 5-HT$_6$ receptor is the most recently discovered and cloned member of the serotonin receptor family, which comprises currently a total of 14 distinct receptors with a variety of different functions and diverse localization patterns. It is positively coupled to adenylate cyclase and rather unique in its structure, exhibiting only 30−40% sequence homology versus all the other serotonin receptor subtypes. Immunological methods revealed high levels of expression in the olfactory tubercle, striatum, frontal cortex, and hippocampus with almost no localization in the periphery.[1−7]

Though a variety of antipsychotics such as clozapine, as well as classical antidepressants, are potent antagonists of this serotonin receptor subtype, its physiological relevance is still under debate.[8] Treatment of rats with antisense oligonucleotides evoked behavioral syndromes, which could only be antagonized by atropine indicating a possible involvement of 5-HT$_6$ receptors in the modulation of cholinergic neurotransmission.[9] These findings were confirmed by use of the first selective 5-HT$_6$ receptor antagonist, RO-04-6790.[9,10]

During the past few years, the discovery and development of a series of novel ligands for the 5-HT$_6$ receptor has been reported, introducing various new classes of compounds as potent and selective binders for this serotonin receptor subtype.[11−15]

The in vivo pharmacology of these ligands and their relevance for the treatment of CNS-related disorders has been discussed and summarized in detail in a series of reviews.[16]

In vivo microdialysis studies in freely moving rats with SB-271046,[12] a potent (pK_i 8.9) and selective 5-HT$_6$ receptor antagonist, revealed a significant increase of the excitatory neurotransmitters aspartate and glutamate in the frontal cortex and hippocampus at doses of 10 mg/kg sc.[17] The observed increase of glutamate in the frontal cortex has been substantially diminished by coinfusion of the voltage dependent Na^+ channel blocker tetrodotoxin (10 μM) and not by coadministration of the muscarinic antagonist atropine (3 mg/kg sc), indicating a tonic serotonergic modulation of glutamatergic neurons via the 5-HT$_6$ receptor without a direct participation of cholinergic pathways.[18]

Recently we reported on the optimization and biological evaluation of *N*-heteroaryl and *N*-aryl sulfonamides as 5-HT$_6$ receptor selective antagonists.[19]

Some representatives thereof reversed a scopolamine induced retention deficit in a passive avoidance paradigm with minimal effective doses (MED) below 10 mg/kg po.[20]

To further optimize these compounds and simultaneously extend the structural scope of 5-HT$_6$ receptor ligands, the synthesis and evaluation of corresponding sulfone congeners has been anticipated. In a very focused approach based on the SAR derived from our sulfonamide series, a limited number of sulfone analogues have been synthesized and investigated.[21] The pyridyl sulfone derivative **2** exhibited a 10-fold higher affinity for the 5-HT$_6$ receptor as compared to its sulfonamide analogue **1**,[19] indicating a significantly improved recognition by the receptor for sulfones as opposed to sulfonamides. The superb selectivity profile

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† Dedicated to Prof. Dr. A. I. Meyers on the occasion of his 70th birthday.

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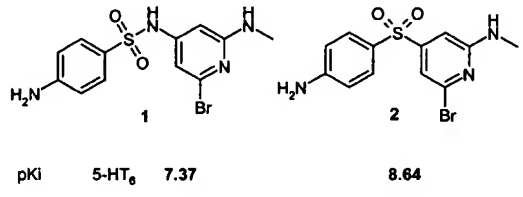
Table 1. Serotonin Subtype Receptor Affinities of Sulfones **2**, **10**–**12**[a]

compd	Y	pK_i (±SEM) 5-HT$_6$	5-HT$_{1D}$	5-HT$_{2A}$	5-HT$_{2C}$	5-HT$_7$
2	NHMe	8.64 ± 0.01	<5	nd	<5	<5
10	Br	7.27 ± 0.02	nd	nd	nd	nd
11	pyrrolidinyl	9.00 ± 0.02	<4	5.84	<5	<4
12	piperazinyl	9.94 ± 0.02	5.95	nd	7.69	<5
	Ro 04-6790[11]	7.26 ± 0.06	<5	<5	<5	<5

[a] The following receptors and radioligands were used in the binding assays: 5-HT$_6$ (human recombinant receptors expressed in HeLa cells, [^{3}H]-LSD); 5-HT$_{1D}$ (human recombinant receptors expressed in HEK 293 cells, [^{3}H]-LSD); 5-HT$_{2A}$ (human recombinant receptors expressed in 3T3 cells, [^{3}H]-DOB); 5-HT$_{2C}$ (human recombinant receptors expressed in 3T3 cells, [^{3}H]-5-HT); 5-HT$_7$ (human recombinant receptors expressed in CHO cells, [^{3}H]-LSD).[11] 5-HT6 results are the mean ± SEM of the three independent determinations performed in triplicate. Selectivity data presented versus other serotonin receptor subtypes are screening data.

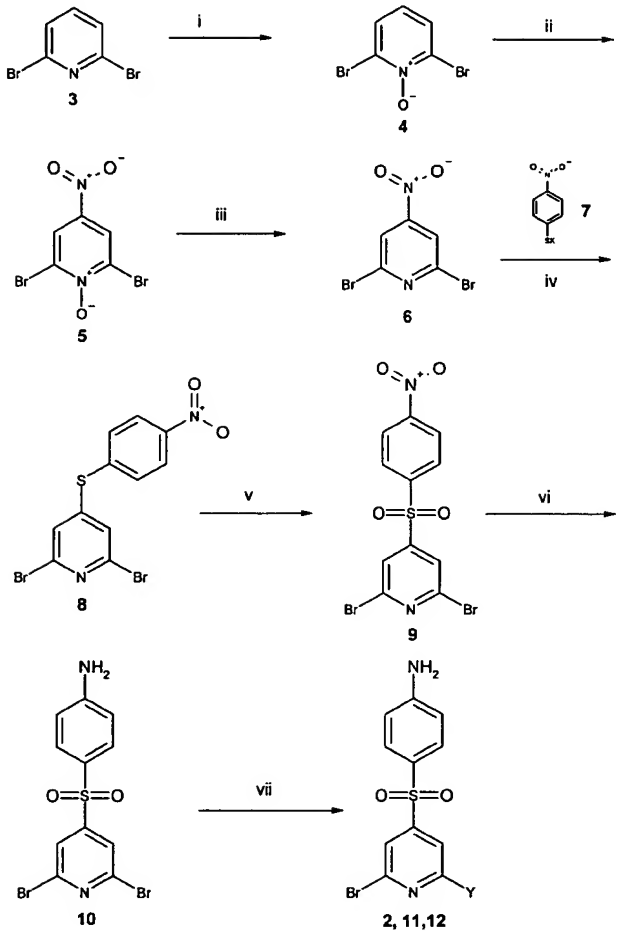
of **2**, at least 1000-fold selectivity over 5-HT$_{1D,2C,7}$ receptors (Table 1), corroborated our new approach even more.



The synthetic approach to this series was based on the known 2,6-dibromo-4-nitropyridine *N*-oxide **5** as a key intermediate (Scheme 1). Commercially available 2,6-dibromopyridine **3** was converted into the 4-nitro *N*-oxide **4** as described by den Hertog.[22,23] Chemoselective reduction with PBr_3 in CH_3CN under reflux conditions for 14 h yielded 2,6-dibromo-4-nitropyridine **6** quantitatively.[24] Nucleophilic displacement of the 4-nitro group by 4-nitro-thiophenol potassium salt **7**[25] in DMF led to sulfide **8** in 86% yield. Conversion of the sulfide moiety to the sulfone by means of mCPBA followed by reduction of the nitro group to the 4-amino functionality with Fe and NH_4Cl in H_2O/MeOH gave rise to the dibromo key intermediate **10** in high yield. Replacement of one bromo substituent by several primary and secondary amines yielded a variety of potent 5-HT$_6$ receptor antagonists. The most potent compounds thereof are summarized in Table 1, exhibiting the cyclic amines **11** and **12** as the best substituents for the 5-HT$_6$ receptor. The piperazinyl derivative **12** with picomolar affinity unfortunately displays significant affinity for the 5-HT$_{2C}$ receptor, which can be attributed to the mCPP (*m*-chlorophenylpiperazine)-like substructure of the molecule. Substitution with various other cyclic as well as open chain amines did not result in compounds with improved affinity (data not shown).

Due to its high affinity for the 5-HT$_6$ receptor and high selectivity within the serotonin receptor family (>1000-fold), pyrrolidine **11** has been selected for further profiling. A broad screen revealed no further binding affinity for a subset of more than 50 neuroreceptors and proteins, including muscarinic, purinergic, dopaminergic, opiate, gabaergic, histaminergic, adrenergic, nicotinergic, and tachykinergic receptors, as well

Scheme 1. Synthesis of Pyridyl Sulfones **2** and **10**–**12**[a]



[a] Reagents: (i) H_2O_2 30%, CF_3COOH, 100 °C, 3 h, 79%; (ii) HNO_3/H_2SO_4, 100 °C, 1.5 h, 80%; (iii) PBr3, CH_3CN, reflux, 14 h, 99%; (iv) DMF, 60 °C, 3 h, 86%; (v) mCPBA, CH_2Cl_2, rt, 2 h, 87%; (vi) Fe, NH_4Cl, H_2O/MeOH, reflux, 1.5 h, 80%; (vii) amine, dioxane, rt, 2 h, (70–90%).

as various calcium and potassium ion channels in the submicromolar range (data not shown).

The intrinsic properties of **11** have been assessed in a functional cAMP-binding assay using 5-CT to stimulate human 5-HT$_6$ receptors stably expressed in HEK-293 cells. As it can be seen in Figure 1, increasing concentrations of **11** shifted the 5-CT concentration response curve parallel rightward without affecting the maximal dose effect. Schild analysis yielded a slope of 1.031, confirming competitive antagonism and a pA_2 of 8.5 in good agreement with the binding value (Figure 1, inset). Compound **11** alone exhibited no intrinsic efficacy suggesting a profile as a silent competitive antagonist.

Table 2 summarizes the physicochemical and DMPK properties of **11**. The in vitro clearance in human and rat liver microsomes is low to intermediate (data not shown). The pharmacokinetic profile of **11** was assessed in rats after both oral and intravenous administration. When administered by oral gavage (10 mg/kg, in PEG/PG/WIP 40/40/20), compound **11** was rapidly absorbed, and sustained plasma concentrations were measured over 8 h (C_{max} = 490 ng/mL, T_{max} = 1 h). Bioavailability was around 50%. After intravenous administration of the same dose and formulation, compound **11** showed a

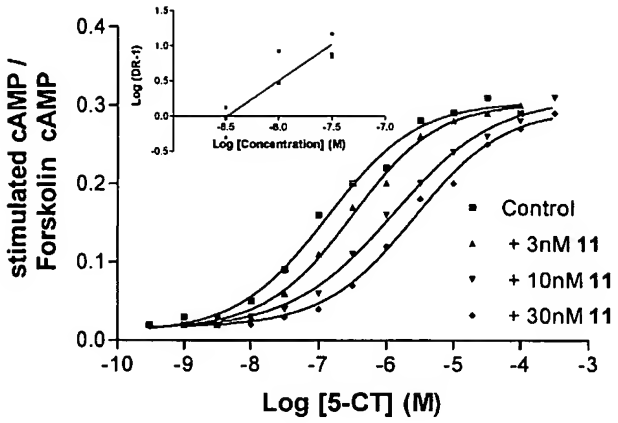


Figure 1. Influence in concentration of compound **11** on 5-CT stimulated adenylate cyclase activity. Inset: Schild analysis of compound **11**, $n = 3$.

Table 2. Physicochemical and Pharmacokinetic Properties of Compound **11**

Physicochemical Properties		
solubility at pH 6.5	log D at pH 7.4	pK_a
1 μg/mL	3.3	<2

Pharmacokinetics in Vivo Rat at 10 mg/kg[a]		
	iv values (average ± SEM)	po values (average ± SEM)
CL_P (mL/min/kg)	20 ± 5	–
$t_{1/2}$ (h)	3.7 ± 1.6	3.0 ± 0.4
F (%)	NA	46 ± 10
V_{ss} (L/kg)	1.4 ± 0.2	NA
brain/plasma ratio %	Nd	24 ± 7

[a] CL_P, plasma clearance; $t_{1/2}$, apparent terminal half-life; F, bioavailability; V_{ss}, volume of distribution at steady-state; NA, not applicable.

low to intermediate systemic plasma clearance (in agreement with the in vitro data) and an apparent terminal half-life of 4 h.

CNS penetration studies were also performed with compound **11** in rats after oral administration. According to log D (3.3) and pK_a values (<2) a reasonable brain penetration could have been anticipated (Table 2).

To establish a direct link between 5-HT_6 receptors and cholinergic neurotransmission in the frontal cortex, microdialysis studies in rats have been conducted with **11**.[26]

Oral administration of **11** at the dose of 30 mg/kg produced a clear 2-fold increase of the extracellular level of acetylcholine (ACh) in the rat frontal cortex (Figure 2). The cortical extracellular levels of ACh were maximally increased 20–40 min after administration of **11** and, thereafter, slowly declined and returned to basal levels 2 h after administration. The rapid onset of action of **11** confirms that this compound is rapidly absorbed and able to readily enter the brain, which is in agreement with our assumptions derived from its physicochemical properties (cf. Table 2). **11** did not modify the cortical levels of choline, the precursor for the synthesis of ACh.

The ability of **11** to increase ACh suggests that 5-HT_6 receptors mediate an inhibitory serotoninergic input to the cholinergic inervation of the frontal cortex. Blockade of 5-HT_6 receptors by **11** reduces such inhibitory input.

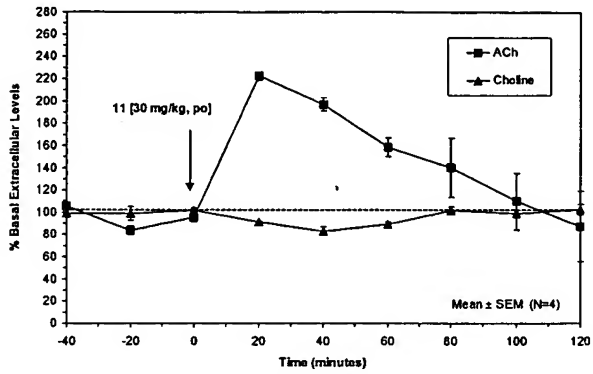


Figure 2. Compound **11** increases the extracellular levels of acetylcholine (ACh), but not choline, in the rat frontal cortex. Each point represents mean ± SEM of 4 animals.

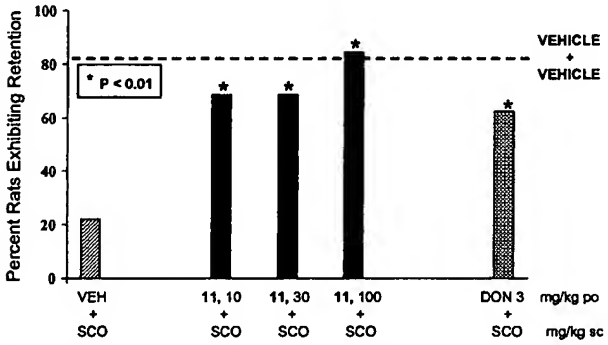


Figure 3. Reversal of a scopolamine (SCO)-induced retention deficit in a passive avoidance task by a single post-training oral administration of either compound **11** (solid bar) or donepezil (DON) in comparison with vehicle (VEH).

This release of ACh is in good agreement with the activity of 5-HT_6 receptor antagonists in reversing a scopolamine-induced passive avoidance retention deficit in rats.[19,20] Compound **11** was tested under both conditions of acute and repeated treatment. In these experiments, the reference AChE inhibitor donepezil was included as an active control condition.

Following a single oral administration, **11** was found to statistically significantly reverse a scopolamine-induced passive avoidance retention deficit at 10–100 mg/kg (Figure 3). In a subsequent experiment in which the treatments were administered orally on 10 successive days followed by evaluation for reversal of a scopolamine-induced passive avoidance deficit, 3 and 10 mg/kg of **11** exhibited a significant ameliorative effect compared to the vehicle condition (Figure 4). In both the acute and chronic experiments, the optimal effect of **11** was approximately that achieved with a single optimal dose of the reference AChE inhibitor donepezil.

Aryl pyridyl sulfone **11** proved to be a highly potent and selective 5-HT_6 receptor antagonist with a very good overall DMPK profile allowing for its use as an ideal pharmacological tool in the elucidation of the functional role of this particular serotonin receptor subtype in a variety of in vivo studies. By means of in vivo microdialysis studies in the frontal cortex and in a passive avoidance paradigm a potential relevance of the 5-HT_6 receptor for cognition and memory related effects has been shown. These experimental results contribute further evidence supporting the therapeutic potential

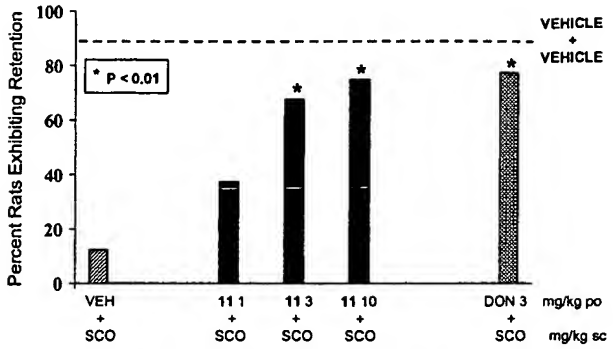


Figure 4. Reversal of a scopolamine (SCO)-induced retention deficit in a passive avoidance task by repeated daily oral administration of either compound **11** (solid bar) or donepezil (DON) in comparison with vehicle (VEH).

for 5-HT_6 receptor antagonists for the treatment of memory disorders.

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Supporting Information Available: Experimental procedures and spectral characterization data for compounds **2** and **10**–**12** are available free of charge via the Internet at http://pubs.acs.org.

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SPECIAL REPORT

In vivo effects of the 5-HT_6 antagonist SB-271046 on striatal and frontal cortex extracellular concentrations of noradrenaline, dopamine, 5-HT, glutamate and aspartate

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Although the 5-HT_6 receptor subtype was identified some 5 years ago, very little is known about its function within the brain. Here we demonstrate, for the first time, the neurochemical effects of a selective 5-HT_6 receptor ligand. Using *in vivo* microdialysis in the freely moving rat, we evaluated the effects of the selective 5-HT_6 receptor antagonist SB-271046 by simultaneous measurement of 5-hydroxytryptamine (5-HT), dopamine (DA), noradrenaline (NA), glutamate and aspartate from the striatum and frontal cortex. SB-271046 did not alter basal levels of 5-HT, DA and NA in either brain region. Similarly, there was no change basal levels of either of the excitatory amino acids within the striatum. In contrast, administration of SB-271046 (10 mg kg^{-1} s.c.) produced a significant ($P<0.05$), tetrodotoxin-dependent, increase in extracellular levels of both glutamate and aspartate within the frontal cortex, reaching maximum values of 375.4±82.3 and 215.3±62.1% of preinjection values, respectively.
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Keywords: 5-HT_6 receptor; microdialysis; 5-HT; dopamine; noradrenaline; glutamate; aspartate; frontal cortex; striatum; SB-271046

Abbreviations: 5-HT, 5-hydroxytryptamine (serotonin); DA, dopamine; NA, noradrenaline; Glu, glutamate; Asp, aspartate; TTX, tetrodotoxin

Introduction The most recently identified of the 5-hydroxytryptamine (5-HT, serotonin) receptors is the 5-HT_6 receptor. Initially cloned from rat striatum (Monsma *et al.*, 1993; Ruat *et al.*, 1993), the 5-HT_6, like 5-HT_4 and 5-HT_7, is a G protein-coupled receptor, which stimulates adenylate cyclase *via* G_s. *In situ* hybridization and Northern blot studies have revealed that 5-HT_6 receptor mRNA appears to be almost exclusively expressed within the brain (Monsma *et al.*, 1993; Ruat *et al.*, 1993). Although 5-HT_6 receptor expression is widespread the highest levels are found within the olfactory tubercle, striatum, nucleus accumbens, cerebral cortex, dentate gyrus and CA subfields of the hippocampus (Monsma *et al.*, 1993; Ruat *et al.*, 1993; Ward *et al.*, 1995). These studies have also revealed that 5-HT_6 receptor is present within 5-HT projection fields and not in the 5-HT neurones of the raphe, indicating a probable postsynaptic role for this receptor (Ward *et al.*, 1995).

The exact functional role of the 5-HT_6 receptor has yet to be ascertained however, its distribution and high affinity (nM) for many antipsychotic and antidepressant drugs, suggests a possible role in both schizophrenia and depression (Monsma *et al.*, 1993; Roth *et al.*, 1994). *In vivo* administration of 5-HT_6 receptor antisense oligonucleotides into the brain demonstrated a behavioural syndrome, which was blocked by the muscarinic antagonist atropine (Bourson *et al.*, 1995). More recently, a number of behavioural studies (Bourson *et al.*, 1998; Bentley *et al.*, 1999) using the selective antagonists Ro 04-6790 have provided additional evidence to suggest a role for the 5-HT_6 receptor in the modulation of cholinergic neurotransmission. This suggests that 5-HT_6 receptor antagonists may also have therapeutic utility in the treatment of memory and cognitive dysfunction.

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Here, we demonstrate for the first time the neurochemical effects of 5-HT_6 antagonism using the potent, selective and bioavailable 5-HT_6 antagonist 5-Chloro-N-(4-methoxy-3-piperazin-1-yl-phenyl)-3-methyl-2-benzothiophenesulfon-amide (SB-271046; Bromidge *et al.*, 1999) and *in vivo* microdialysis in the freely moving rat.

Methods *Microdialysis* Male Sprague-Dawley rats (280–350 g, Charles River) were used in all experiments. Surgical implantation was performed as previously described (Dawson and Nguyen, 1998). Coordinates for the striatum and frontal cortex were: RC +0.2, L −3.0, V −3.0 and RC +3.2, L −3.5, V −1.5 (RC and L from bregma and V coordinates from the skull), respectively. Eighteen to twenty-four hours post surgery pre-equilibrated microdialysis probes were inserted into the striatum or frontal cortex (O.D. 0.5 mm, membrane length 4 mm and 2 mm, respectively; CMA/Microdialysis, Sweden) of the unrestrained rat. Microdialysis sampling was carried out by the method of Dawson and Nguyen (1998) with a perfusion rate of 1.25 μl min^{-1}. SB-271046 or vehicle was administered *via* a surgically implanted s.c. cannula. Tetrodotoxin (TTX; 10 μM; Alamone Labs, Israel) was infused *via* the microdialysis probe for a period of 40 min ($t=180-220$).

Analysis of dialysates Dialysates (25 μl) were split and analysed for excitatory amino acids (5 μl) and monamine/catacholamines (20 μl) as follows: (1) NA, DA and 5-HT were separated by reverse phase high performance liquid chromatography (HPLC) (C18 ODS3 column, 150×3.0 mm, Metachem, Torrance, CA, U.S.A.) and detected using an ANTEC electrochemical detector (ANTEC, Netherlands) set at a potential of 0.65 V vs a Ag/AgCl reference electrode. Mobile phase was delivered by a Jasco PU980 HPLC pump (Jasco Ltd, Essex, U.K.) at

0.5 ml min^{-1} and contained 0.1 M NaH_2PO_4 buffer at pH 4.8, 0.25 mM EDTA, 0.23 mM 1-octane sodium sulphonic acid, 2% isopropanol and 7.5% methanol. (2) Analysis of glutamate and aspartate was performed using a Crystal 310 capillary electrophoresis system (Thermo BioAnalysis, MN, U.S.A.) with a Zeta laser induced fluorescence detector (ZETA Technology, Toulouse, France) by the method of Dawson *et al.* (1997). All data were acquired using the Atlas software package (Thermo Labsystems, Gulph Mills, PA, U.S.A.) for the PC.

Data analysis Results were analysed by analysis of variance with repeated measures followed by pairwise comparisons using Bonferroni adjustment for multiple comparisons using the Statview software application (Abacus Concepts Inc., Berkeley, CA, U.S.A., 1996).

Results *Effects of SB-271046 on extracellular levels of 5-HT, NA, DA, Glu and Asp in the striatum* Subcutaneous injection of 10 mg kg^{-1} SB-271046 produced no significant change in extracellular concentrations of 5-HT, DA, NA, Glu or Asp within the striatum of the freely moving rat (Table 1) for up to 240 min post-administration.

Effects of SB-271046 on extracellular levels of 5-HT, NA, DA, Glu and Asp in the frontal cortex SB-271046 (1 and 10 mg kg^{-1} s.c.) produced no significant change in basal extracellular concentrations of 5-HT or NA within the rat frontal cortex (Figure 1). A small increase in extracellular DA was observed at 10 mg kg^{-1} (Figure 1) but this failed to reach statistical significance. Conversely, SB-271046 (10 mg kg^{-1}) produced a significant ($P<0.05$) increase in extracellular Glu and Asp concentrations reaching maximum values of 375.4±82.3% ($t=280$ min) and 215.3±62.1% ($t=240$ min) of preinjection values, respectively (Figure 2). Infusion of TTX (10 μM) did not significantly change basal levels of either excitatory amino acid (Figure 2). In contrast, following the initial SB-271046-induced increases, infusion of TTX ($t=180-220$ min) resulted in a significant attenuation in extracellular glutamate levels reducing maximal levels to 185±12.4% ($t=260$ min; Figure 2) of preinjection control. Concurrently, TTX infusions produced a significant ($P<0.05$) attenuation in SB-271046-induced increases in aspartate, however this effect seemed to be somewhat delayed ($t=280$; Figure 2).

Discussion These data demonstrate for the first time the *in vivo* neurochemical effects of 5-HT_6 receptor antagonism on basal extracellular concentrations of neurotransmitters. SB-271046 produced no significant change in extracellular concentrations of any of the neurotransmitters measured in the striatum. Similarly, SB-271046 (1 and 10 mg kg^{-1}) produced no significant change in basal levels of NA, DA or

Table 1 Effects of SB-271046 (10 mg kg^{-1} s.c.) on striatal neurotransmitter levels

	Maximum % of preinjection control levels	
	Vehicle treated	*SB-271046 treated*
NA	111.0±24.5	129.0±24.1
DA	96.2±18.8	125.4±19.2
5-HT	102.4±14.9	78.5±16.9
Glu	115.5±11.5	104.3±25.8
Asp	113.3±28.9	111.5±29.7

Data expressed as mean±s.e.mean ($n=8$ per study group). Basal preinjection levels of neurotransmitter within the striatum were: 5-HT−8.8+0.2, NA−37±1.3, DA−38.2 ±0.8 fmol per 20 μl microdialysate and Glu−1.09±0.03, Asp−0.38±0.01 μM ($n=44$). None of these changes attained statistical significance.

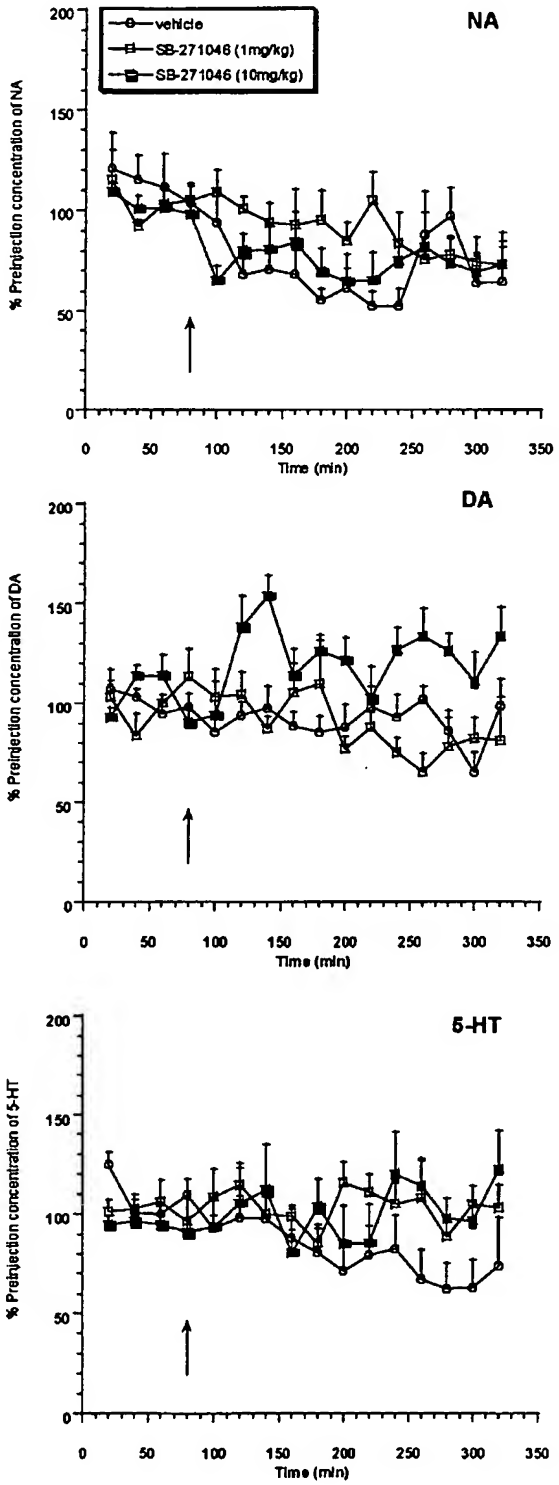

Figure 1 Effects of SB-271046 (1 and 10 mg kg^{-1} s.c.) on frontal cortex extracellular levels of NA, DA and 5-HT. Data expressed as mean±s.e.mean, ($n=7-12$ per study group). Arrow denotes subcutaneous drug or vehicle injection points. Basal preinjection levels of neurotransmitter within the frontal cortex were: 5-HT−9.7±0.2, NA−11.1±0.2, DA−14.1±0.25 fmol per 20 l microdialysate ($n=42$).

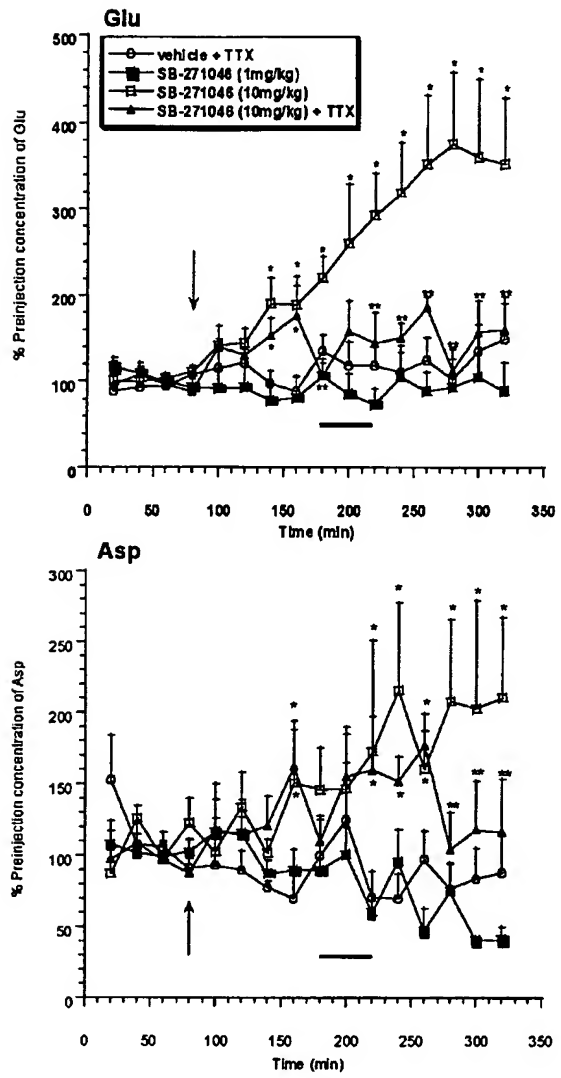

Figure 2 Effects of SB-271046 (1 and 10 mg kg^{-1} s.c.) on frontal cortex extracellular levels of Glu and Asp. Data expressed as mean±s.e.mean, (n=7–12 per study group). Arrow denotes subcutaneous drug or vehicle injection points; solid bar denotes TTX or vehicle infusion. Basal preinjection levels of neurotransmitter within the frontal cortex were: and Glu–1.08±0.02, Asp–0.17±0.01 μM (n=42).*Denotes statistical significance (P<0.05) from vehicle treated animals. **Denotes statistical significance (P<0.05) SB-271046 alone vs SB-271046+TTX treated animals.

5-HT within the frontal cortex. However, 10 mg kg^{-1} of SB-271046 induced a prolonged and robust 3 and 2 fold increase in frontal cortex glutamate and aspartate, respectively. These increases were attenuated *via* the infusion of the voltage dependent Na^+ channel blocker TTX, indicating that these amino acids are released from glutamatergic neurones. Given that the 5-HT_6 receptor is highly expressed in the cortex and basal ganglia structures (Ward *et al.*, 1995), it is likely that the observed enhancement of excitatory transmission originates from a blockade of tonic serotonergic inhibition of intrinsic cortical projections or basal ganglia/thalamocortical connections.

Since the discovery of the 5-HT_6 receptor, evidence has been accumulating to suggest a role for this receptor in the modulation of cholinergic function. Early experiments showed that administration of 5-HT_6 receptor antisense oligonucleotides into the brain induced a behavioural syndrome, which could be blocked by the muscarinic antagonist atropine (Bourson *et al.*, 1995). More recently, Bourson *et al.* (1998) and Bentley *et al.* (1999) have demonstrated that the selective 5-HT_6 receptor antagonist Ro 04-6790 can induce stretching and inhibit 6-OHDA lesion-induced rotational behaviours. Again, both of these behaviours can be blocked by the application of muscarinic antagonists. Moreover, a recent report actually demonstrated increases in extracellular levels of acetylcholine within both the cortex and hippocampus with a similar selective antagonist Ro 65-7199, at doses shown to be effective in behavioural models of memory deficit (Sleight *et al.*, 1999). Routledge *et al.* (1999) showed that SB-271046 could potentiate physostigmine induced chewing behaviours and the same compound has been shown to be effective in enhancing cognitive function in models of learning and memory (Rogers *et al.*, 1999). It therefore seems clear from these data that the 5-HT_6 receptor is playing some role in cholinergic transmission. A direct link between acetylcholine and cortical glutamate has been demonstrated (Consolo *et al.*, 1996; Sanz *et al.*, 1997), therefore, the enhanced cholinergic effects and the observations presented here may be connected but the exact mechanism cannot be fully elucidated at this time.

Interestingly, Stean *et al.* (1999) has reported anticonvulsant properties of SB-271046 at doses much lower than those shown to be effective here or within cognitive studies (Rogers *et al.*, 1999). The mechanism of this anticonvulsant action is not clear, particularly given the enhancement of glutamatergic function. There does seem to be some differential between effective doses in these models (0.1 and 1 mg kg^{-1} minimum effective dose for the electroconvulsive shock and cognitive models, respectively) and the effects observed here. The reason for this maybe due to a difference in the tone experienced by the 5-HT_6 receptor in the various models. Non selective, as would occur in electroconvulsive shock and more selective stimulation, as occurs in models of learning and memory, is likely to bring about elevations in serotonergic tone (although this is not necessarily selective for 5-HT). In a higher tonic environment the effective doses of antagonist required to block the receptor will be somewhat less than in a non-stimulated basal situation when tone is likely to be much lower.

Given this neurochemical profile of a 5-HT_6 antagonist one can speculate on the therapeutic utility of such a compound. Previous findings (Bourson *et al.*, 1995) have suggested a therapeutic role for 5-HT_6 antagonists in the treatment of memory and cognitive dysfunction and more recent data has demonstrated enhanced cognitive function in various preclinical models (Sleight *et al.*, 1999; Rogers *et al.*, 1999). The role of the prefrontal cortex (Koechlin *et al.*, 1999) and more specifically glutamate (Dudkin *et al.*, 1996) in cognition has become evident. Enhancement of excitatory neurotransmission within the frontal cortex may therefore have some utility in the treatment of memory and/or cognitive dysfunction. Atypical antipsychotics such as clozapine, which posses nM affinity for the 5-HT_6 receptor, have also been shown to be somewhat effective in the treatment of the cognitive deficits associated with schizophrenia (Tollefson, 1996). Interestingly, clozapine has also been shown to selectively enhance excitatory transmission within the frontal cortex (Daly & Moghaddam, 1993).

In summary, we demonstrate here, for the first time, the enhancement of excitatory neurotransmission by a 5-HT_6 antagonist. Selective augmentation of extracellular concentrations of both glutamate and aspartate within the frontal cortex by SB-271406 suggests that 5-HT_6 antagonists may have some therapeutic utility in the treatment of cognitive dysfunction.

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ELSEVIER

The 5-HT_6 Receptor Antagonist SB-271046 Selectively Enhances Excitatory Neurotransmission in the Rat Frontal Cortex and Hippocampus

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Preclinical evidence has suggested a possible role for the 5-HT_6 receptor in the treatment of cognitive dysfunction. However, currently there is little neurochemical evidence suggesting the mechanism(s) which may be involved. Using the selective 5-HT_6 antagonist SB-271046 and in vivo microdialysis, we have evaluated the effects of this compound on the modulation of basal neurotransmitter release within multiple brain regions of the freely moving rat. SB-271046 produced no change in basal levels of dopamine (DA), norepinephrine (NE) or 5-HT in the striatum, frontal cortex, dorsal hippocampus or nucleus accumbens. Similarly, this compound had no effect on excitatory neurotransmission in the striatum or nucleus accumbens. Conversely, SB-271046 produced 3- and 2-fold increases in extracellular glutamate levels in both frontal cortex and dorsal hippocampus, respectively. These effects were completely attenuated by infusion of tetrodotoxin but unaffected by the muscarinic antagonist, atropine. Here we demonstrate for the first time the selective enhancement of excitatory neurotransmission by SB-271046 in those brain regions implicated in cognitive and memory function, and provide mechanistic evidence in support of a possible therapeutic role for 5-HT_6 receptor antagonists in the treatment of cognitive and memory dysfunction.
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KEY WORDS: *5-HT_6 receptor; SB-271046; Microdialysis; Glutamate; Striatum; Frontal cortex; Dorsal hippocampus; Nucleus accumbens; Cognition*

The 5-hydroxytryptamine (5-HT, serotonin) receptor superfamily currently consists of 14 members divided into seven classes (5-HT_{1-7}) according to structural and functional homologies (for review see Barnes and Sharp 1999). One of the most recently identified of these is the 5-HT_6 receptor. Initially cloned from rat striatum (Monsma et al. 1993; Ruat et al. 1993), the 5-HT_6 receptor, like 5-HT_4 and 5-HT_7, is a G protein-linked receptor, which stimulates adenylate cyclase via G_s – coupling. *In situ* hybridization and Northern blot studies have revealed that 5-HT_6 receptor mRNA appears to be almost exclusively expressed within the brain (Monsma et al. 1993; Ruat et al. 1993; Ward et al. 1995). Regional analysis of expression reveals that the highest levels of mRNA are found within the olfactory tubercle, striatum, nucleus accumbens, cerebral cortex and subfields of the hippocampus (Monsma et al. 1993; Ruat et al. 1993; Gerard et al. 1996). Similarly, Gerard et al. (1997) showed a comparable distribution of protein using polyclonal antibodies raised to a presumed unique portion of the C terminus of the receptor. These studies also revealed that 5-HT_6 receptor expression appears to be present within 5-HT projection fields and not in 5-HT neurons

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of the raphe, indicating a probable postsynaptic role for this receptor (Ward et al. 1995; Gerard et al. 1997).

The exact functional role of the 5-HT_6 receptor has yet to be ascertained; however, its distribution together with its high affinity (nM) for many of the therapeutically effective antipsychotic and antidepressant drugs suggests possible therapeutic roles in both schizophrenia and depression (Monsma et al. 1993; Roth et al. 1994). Early experiments showed that administration of antisense oligonucleotides into the brain induced a behavioral syndrome, which could be blocked by the muscarinic antagonist, atropine (Bourson et al. 1995). A further study showed that antisense oligonucleotide treatment failed to alter the gross behavior of animals during a conditioned fear stress paradigm (a model of anxiety), but an attenuation of anxiety-induced prefrontal cortex 5-HT release was observed (Yoshioka et al. 1998). 5-HT_6 knockout mice have also been reported and these animals do not appear to have any marked phenotypic abnormalities, but do display some increase in anxiety in the elevated zero-maze (Tecott et al. 1998). More recently, Bentley et al. (1999) and Bourson et al. (1998) have demonstrated that the selective antagonist Ro 04-6790 can induce stretching and inhibit 6-OHDPAT lesion-induced rotational behaviors. Both these phenomena can be blocked by the application of muscarinic antagonists. Routledge et al. (1999) observed a potentiation of physostigmine-induced chewing behavior by SB-271046 and the same compound has been shown to be effective in enhancing cognitive function in models of learning and memory (Rogers et al. 1999). Taken together these data indicate that the 5-HT_6 receptors may be involved in the modulation of cholinergic function, suggesting a possible therapeutic utility in the treatment of memory and cognitive dysfunction.

Although learning and memory has been suggested as a therapeutic target, very little neurochemical data has been reported in support of this hypothesis. A recent abstract communication demonstrated increases in extracellular levels of acetylcholine within both the cortex and hippocampus following administration of the selective antagonist Ro 65-7199, at doses shown to be effective in behavioral models of memory deficit (Sleight et al. 1999). Furthermore, we have recently reported preliminary observations demonstrating increases in extracellular levels of glutamate within the frontal cortex by SB-271046 (Dawson et al. 2000). Therefore, to more fully elucidate the neurochemical mechanism responsible for the observed cognitive enhancement, we have examined the role of the 5-HT_6 receptor in the modulation of multiple neurotransmitters in those brain regions shown to have the highest receptor expression levels (Monsma et al. 1993; Ruat et al. 1993; Ward et al. 1995; Gerard et al. 1997). Using the potent, selective and bioavailable 5-HT_6 antagonist 5-Chloro-N-(4-methoxy-3-piperazin-1-yl-phenyl)-3-methyl-2-benzothiophenesulfon- amide (SB-271046; Bromidge et al. 1999) and *in vivo* microdialysis, we have evaluated the effects of this compound on the modulation of basal 5-HT, dopamine (DA), norepinephrine (NE) and glutamate (Glu) release within multiple brain regions in the freely moving rat.

METHODS

Materials

All chemicals used were analytical grade and were purchased from Aldrich & Sigma chemicals (Milwaukee, WI, USA). Tetrodotoxin (TTX) was purchased from Alamone labs (Jerusalem, Israel). Atropine was purchased from Research Biochemical International (Natick, MA, USA). (5-Chloro-N-(4-methoxy-3-piperazin-1-yl-phenyl)-3-methyl-2-benzothiophenesulfonamide (SB-271046) was synthesized by Chemical Sciences, Wyeth Ayerst Research (Princeton, NJ, USA).

Animals

Male Sprague-Dawley rats (280–350 g, Charles River Laboratories, Wilmington, MA) were used in all experiments (n = 8–14 per study group). Animals were group housed in cages with food and water available *ad libitum*. Following surgery, the animals were singly housed in Plexiglass cages (45 × 45 × 30 cm) with food and water available *ad libitum*. All animal studies were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institute of Health.

Surgical procedure

Following induction of anesthesia, with gaseous administration of halothane (3%) (Fluothane, Zeneca, Cheshire, UK), the animals were secured in a stereotaxic frame with ear and incisor bars. Anesthesia was maintained by continuous administration of halothane (1–2%). A microdialysis probe guide cannula (CMA/Microdialysis, Stockholm, Sweden) was implanted into either the striatum (RC +0.2, L–3, V–3), frontal cortex (RC+3.5, L-3.2, V-1.5), dorsal hippocampus (RC-4.3, L-2.6, V-2.1) or nucleus accumbens (RC+2.2, L-1.4, V-6.0). Co-ordinates were taken according to Paxinos and Watson (1986) with reference points taken from bregma and vertical from the skull. A subcutaneous cannula (s.c.) was also implanted at this time between the animal's shoulders. Both cannula were secured to the skull using dental acrylic (Plastics One, Roanoke, VA, USA). The wound was sutured and the animals left to recover for 18–24 h in their home cages with free access to food and water.

Microdialysis

A pre-equilibrated microdialysis probe was inserted into the guide cannula, in either the striatum (O.D. 0.5 mm, membrane length 4 mm; CMA/Microdialysis, Sweden), frontal cortex, dorsal hippocampus or nucleus accumbens (O.D. 0.5 mm, membrane length 2 mm; CMA/Microdialysis, Sweden) of the unrestrained rat, post surgery. The probe was perfused with artificial cerebrospinal fluid (aCSF; NaCl 125 mM, KCl 3.0 mM, $MgSO_4$ 0.75 mM, $CaCl_2$ 1.2 mM and 0.1 M phosphate buffer pH 7.4) at a flow rate of 1.0 μl/min. A 3 h stabilization period was allowed following probe implantation, after which time microdialysis sampling was carried out by a modification of the method of Dawson and Nguyen (1998). Four baseline samples were taken prior to drug injection to achieve a steady baseline. These four samples were averaged and all subsequent values were expressed as a percentage of this preinjection value. SB-271046 (10 mg/kg), atropine (3 mg/kg) or vehicle was administered via the s.c. cannula. TTX (10μM) was infused, via reverse microdialysis, through the probe for a period of 40 min (t = 180–220). A 20-min sampling regime was used throughout the experimental period. At the end of the experiment probe placement was verified histologically and data from animals with incorrect probe placement were discarded.

Analysis of Microdialysates

Microdialysates were split and taken for amino acid analysis and monoamine/catacholamine determinations as follows:

1) NE, DA and 5-HT were separated by reverse phase high performance liquid chromatography (HPLC) (C18 ODS2 column, 100 x 3.0 mm, Metachem, Torrance, CA, USA) and detected using an ANTEC electrochemical detector (ANTEC, Netherlands) set at a potential of 0.7V vs an Ag/AgCl reference electrode. Mobile phase was delivered by a Jasco PU980 HPLC pump (Jasco Ltd, Essex, UK) at 0.5 ml/min and contained 0.15 M NaH_2PO_4 buffer at pH 4.3, 0.25 mM EDTA, 1.5 mM 1-octane sodium sulphonic acid and 5% isopropanol.

2) Measurement of glutamate was performed using a Crystal 310 capillary electrophoresis system (Thermo BioAnalysis, NM, USA) with a Zeta laser induced fluorescence detector (ZETA Technology, Toulouse, France) coupled with a Helium-Cadmium laser (Em-442 nm; Omnichrome, CA, USA). All samples were pre-derivatized with naphthalene 2,3-dicarboxaldehyde (NDA) by a modification of the method of Hernandez et al. (1993). Dialysate or standard samples (3 μl) were mixed with 50 mM boric acid buffer pH 9.5 containing 20 mM sodium cyanide (5 μl) and 30 mM NDA in methanol (1 μl). Samples were allowed to react for 3 min at room temperature prior to injection. Separations were performed according to Dawson et al. (1997) in fused silica capillaries (75 μm id, 375 μm od, 47 cm; Polymicro technologies, NM, USA) with an applied voltage of 0.6 kV/cm. Samples (5 nl) were applied to the capillary via a high pressure injection system. Separations used 30 mM boric acid pH 9.5 (pH adjusted using 1 M NaOH). The capillary was rinsed with 0.1 M NaOH (1.5 min) and running buffer (1.5 min) between analyses.

All data were acquired using the Atlas software package (Thermo Labsystems, Gulph Mills, PA) for the PC.

Data Analysis

The fmol/μM perfusate values of transmitters/amino acids for the first four baseline samples were averaged and this value denoted as 100%. Subsequent sample values were expressed as a percentage of this preinjection control value. Results were analyzed by analysis of variance with repeated measures followed by pairwise comparisons using Bonferroni adjustment for multiple comparisons using the Statview software application (Abacus Concepts Inc., Berkeley, CA 1996) for the PC.

RESULTS

Effects of SB-271046 on Extracellular Levels of 5-HT, NE and DA in the Striatum, Frontal Cortex, Dorsal Hippocampus or Nucleus Accumbens of the Freely Moving Rat

Subcutaneous injection of 10 mg/kg SB-271046 produced no significant change in extracellular concentrations of 5-HT, NE or DA within the striatum, frontal cortex, dorsal hippocampus or nucleus accumbens of the freely moving rat (Table 1) for up to 240 min post-administration. A small increase in extracellular DA was observed in the frontal cortex (Table 1); however, this effect failed to reach statistical significance.

Effects of SB-271046 on Extracellular Levels of Glutamate in the Striatum, Frontal Cortex, Dorsal Hippocampus or Nucleus Accumbens of the Freely Moving Rat

Subcutaneous injection of 10 mg/kg SB-271046 produced no significant change in extracellular concentrations of glutamate in the striatum (Table 1). In contrast, the same dose of SB-271046 induced a significant ($p < .05$) increase in extracellular glutamate concentrations in both the frontal cortex and the dorsal hippocampus reaching a maximum values of 375.4 ± 82.3 % (t = 280 min) and 217.8 ± 34.8 % (t = 280 min) of preinjection values, respectively (Table 1). A smaller increase in extracellular glutamate was also observed in the nucleus

Table 1. Effects of SB-271046 (10 mg/kg s.c.) on Extracellular Levels of Neurotransmitters in Various Brain Regions. Data Expressed as Maximum Observed % of Preinjection Levels Mean ± S.E.M. (n = 8–14 Per Group). Also Shown are the Basal Levels of NE, DA and 5-HT (Expressed as Mean ± S.E.M. fmol/10 μl Microdialysate) and Glu (Expressed as Mean ± S.E.M. μM/Microdialysate) from Each Brain Region. * Denotes Statistical Difference ($p < 0.05$) between SB-271046 and Vehicle Treated Animals

	NE	DA	5-HT	Glu
Frontal Cortex				
Basal	11.1 ± 0.2	14.1 ± 0.25	9.7 ± 0.2	1.08 ± 0.02
Vehicle	97.0 ± 14.3	102.1 ± 6.7	98.2 ± 11.0	99.2 ± 17.5
SB-271046	96.9 ± 28.7	133.2 ± 22.9	103.5 ± 14.8	375.4 ± 82.3*
Striatum				
Basal	37.0 ± 1.3	38.2 ± 0.8	8.8 ± 0.2	1.09 ± 0.03
Vehicle	111.0 ± 24.5	96.2 ± 18.8	102.4 ± 14.9	115.5 ± 11.5
SB-271046	129.0 ± 24.1	125.4 ± 19.2	78.5 ± 16.9	104.3 ± 25.8
Hippocampus				
Basal	3.44 ± 0.23	4.6 ± 0.27	13.8 ± 1.37	1.87 ± 0.12
Vehicle	103.6 ± 25.1	108.7 ± 14.5	104.4 ± 27.2	98.8 ± 20.3
SB-271046	142.2 ± 19.2	113.8 ± 13.2	100.2 ± 27.9	217.8 ± 34.8*
N. Accumbens				
Basal	15.5 ± 1.30	33.83 ± 2.0	5.86 ± 0.37	0.52 ± 0.02
Vehicle	106.2 ± 31.5	103.7 ± 9.9	92.4 ± 26.2	124.7 ± 5.60
SB-271046	122.1 ± 25.5	97.8 ± 2.7	134.4 ± 25.9	175.9 ± 24.8

accumbens; however, this effect failed to reach statistical significance (Table 1).

Effects of Tetrodotoxin (10 μM) and Atropine (3 mg/kg s.c.) on SB-271046-induced Increases in Extracellular Glutamate in the Frontal Cortex and Dorsal Hippocampus

Infusion of the voltage-dependent Na^+ channel blocker, tetrodotoxin (TTX; 10 μM), produced no change in basal levels of excitatory amino acid in either brain region examined (Figures 1 and 2). Alternatively, following the initial SB-271046-induced increases, infusion of TTX (t = 180–220 min) resulted in a significant attenuation in extracellular glutamate levels reducing maximal levels to 185.0 ± 12.4 % (t = 260 min; Fig. 1) and 125.5 ± 26.5 % (t = 280 min; Fig. 2) for the frontal cortex and hippocampus, respectively.

Administration of the muscarinic antagonists, atropine (3 mg/kg s.c.) had no significant effect on basal levels of glutamate in either the frontal cortex (Figure 3) or dorsal hippocampus (Figure 4). Similarly, atropine produced no significant change in SB-271046-induced increases in glutamate in either brain region (Figures 3 and 4).

DISCUSSION

The 5-HT_6 receptor is the most recently identified of the 5-HT receptor subtypes (Monsma et al. 1993; Ruat et al. 1993) and although the exact role of this receptor has not been fully elucidated, evidence is accumulating to suggest possible functions. Much of the early data, involving administration of antisense oligonucleotides and more recently with the development of selective antagonists, have suggested a role for the 5-HT_6 receptor in the modulation of cholinergic function. These data, taken together with behavioral observations demonstrating utility in models of cognitive impairment (Rogers et al. 1999; Sleight et al. 1999), have lead to the hypothesis that 5-HT_6 antagonists may have therapeutic utility in the treatment of cognitive and memory

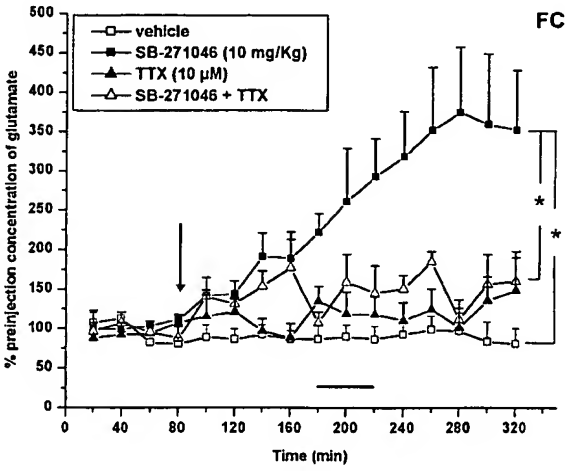

Figure 1. Effects of SB-271046 (10 mg/kg s.c) on extracellular levels of glutamate in the frontal cortex of the freely moving rat. Data expressed as mean ± S.E.M, (n = 8–14 per study group). Arrow denotes subcutaneous drug or vehicle injection points; solid bar denotes TTX (10 μM) or vehicle infusion. * denotes statistical significance ($p < .05$) between groups.

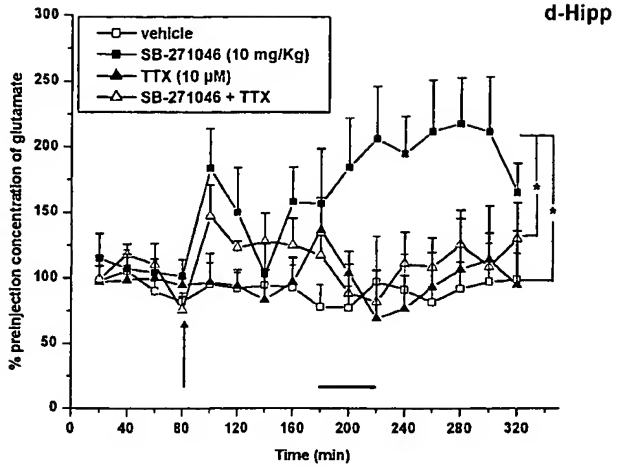

Figure 2. Effects of SB-271046 (10 mg/kg s.c) on extracellular levels of glutamate in the dorsal hippocampus of the freely moving rat. Data expressed as mean ± S.E.M, (n = 8–14 per study group). Arrow denotes subcutaneous drug or vehicle injection points; solid bar denotes TTX (10 μM) or vehicle infusion. * denotes statistical significance ($p < .05$) between groups.

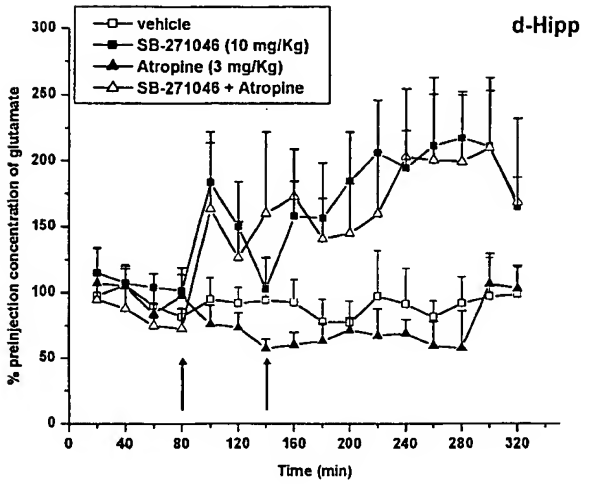

Figure 4. Effects of atropine (3 mg/kg s.c.) on SB-271046 (10 mg/kg s.c.) induced increases extracellular levels of glutamate within the dorsal hippocampus. Data expressed as mean ± S.E.M, (n = 8–14 per study group). Arrow denotes subcutaneous drug or vehicle injection points. * denotes statistical significance ($p < .05$) between groups.

dysfunction. However, very little neurochemical data has been reported to suggest the neurochemical mechanism behind these improvements in cognitive function. Using the selective antagonist SB-271046 (Bromidge et al. 1999) and *in vivo* microdialysis, we have examined the effects of 5-HT_6 receptor blockade on the release of monoamine/catacholamine and excitatory amino acid neurotransmitters in those brain regions demonstrated to have the highest 5-HT_6 receptor expression (Monsma et al. 1993; Ruat et al. 1993). SB-271046 produced no change in basal levels of either 5-HT, DA or NE in any brain region examined, indicating that under these experimental conditions the 5-HT_6 receptor is exerting no tonic control over the release of the conventional monoamine/catacholamine neurotransmitters. Alternatively, however, SB-271046 did produce significant 2- and 3-fold increases in the extracellular concentrations of the excitatory neurotransmitter glutamate, in both the hippocampus and frontal cortex, respectively.

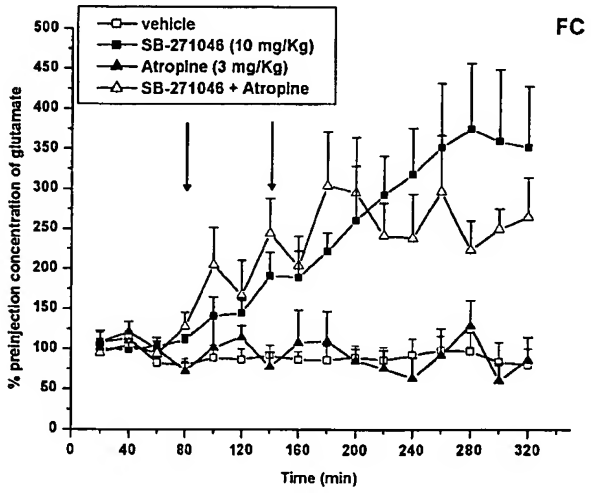

Figure 3. Effects of atropine (3 mg/kg s.c.) on SB-271046 (10 mg/kg s.c.) induced increases extracellular levels of glutamate within the frontal cortex. Data expressed as mean ± S.E.M, (n = 8–14 per study group). Arrow denotes subcutaneous drug or vehicle injection points. * denotes statistical significance ($p < .05$) between groups.

This is the first reported demonstration of the selective enhancement of excitatory neurotransmission by a 5-HT_6 antagonist within those brain regions which are thought to be critical in the control of cognitive and memory processes.

In order to confirm the neuronal origin of the 5-HT_6 receptor antagonist-induced increases in glutamate, we infused the voltage-dependent Na^+ channel blocker, tetrodotoxin (TTX), following SB-271046 administration. TTX infusion attenuated the SB-271046-induced increases in extracellular glutamate in both the frontal cortex and hippocampus, thus indicating that the increases in extracellular excitatory amino acid originate from glutamate neurons in an impulse dependent manner. It would therefore appear that there is a tonic serotonergic inhibition of glutamate neurons exerted either directly or indirectly via the 5-HT_6 receptor. At this time we can only speculate which glutamatergic projection systems are involved, based on reported 5-HT_6 localization (Monsma et al. 1993; Ruat et al. 1993). One possibility is that the enhanced transmission simply

originates from intrinsic glutamatergic neurons within both the cortex and hippocampus. Alternatively, the projection pathways of the basal ganglia/thalamocortical pathways may also be involved in those effects observed in the frontal cortex and perforant pathway and/or the amygdala/fornix connections may also be contributing to the effects in the hippocampus.

Since the 5-HT_6 receptor has been implicated in the modulation of cholinergic function (Bourson et al. 1995; Sleight et al. 1999; Bentley et al. 1999; Routledge et al. 1999) and the selective antagonist, Ro 65-7199, has been demonstrated to increase acetylcholine within both the cortex and hippocampus (Sleight et al. 1999), the influence of the cholinergic system on the observed glutamate effects was examined. Administration of muscarinic antagonist, atropine, at a dose that has previously been shown to block 5-HT_6 antisense oligonucleotide-induced behaviors, had no effect on the SB-271046-induced increases in extracellular glutamate in either brain region, thus indicating that the enhanced excitatory neurotransmission observed was not a consequence of an enhanced cholinergic function. Gerard et al. (1997) has suggested that 5-HT_6 receptors may be expressed on GABAergic spiny neurons and a very recent report (Woolley et al. 2000) actually showed co-localization of glutamic acid decarboxylase (GAD) immunoreactivity with 5-HT_6 receptors within multiple brain regions. Taken together, these data suggest that enhancement of glutamatergic function is not via a direct blockade of tonic serotonergic inhibition of glutamate neurons but is more likely to be an indirect action via the blockade of 5-HT_6 receptors on GABAergic interneurons either within, or on projection pathways to, the hippocampus and cortex. Whether the reported cholinergic effects (Sleight et al. 1999) are a consequence of this increase in glutamate or are an independent event (also mediated via GABA) cannot be determined from these experiments; however, interplay between the two systems has been demonstrated (Consolo et al. 1996; Sanz et al. 1997) and cannot be ruled out.

Interestingly, Routledge et al. (2000) has reported anticonvulsant properties of SB-271046 at doses much lower than those shown to be effective here and in other models (Rogers et al. 1999; Routledge et al. 1999; Dawson et al. 2000). The differences in effective dose may simply be due to the experimental paradigm employed. Electroconvulsive shock will not only increase the animal's serotonergic tone but also non-selectively stimulate multiple other transmitter systems. In contrast, microdialysis experiments are performed under resting conditions when there is little or no external stimulation; thus the endogenous serotonergic tone is likely to be much lower. However, the mechanism of this anticonvulsant action is not clear at this time, particularly, in light of enhanced basal glutamatergic neurotransmission and the hypothesized decrease in GABAergic input.

Since 5-HT_6 receptors are largely located in limbic regions and have nanomolar affinities for the atypical antipsychotic drugs, such as clozapine, it has been speculated that this receptor may have some involvement in schizophrenia. Deficits in glutamatergic function have been suggested to be causal in the cognitive and memory dysfunction observed in psychiatric patients (Hirsch et al. 1997; Breier 1999) and a number of atypical antipsychotics have been shown to be effective in the treatment of the cognitive deficits associated with schizophrenia (Tollefson 1996). A recent report by Healy and Meador-Woodruff (1999) provided direct evidence for a link between glutamatergic systems and 5-HT_6 receptors by showing that blockade of ionotropic glutamate receptors leads to a decrease in 5-HT_6 mRNA expression in various brain regions. Furthermore, evidence suggests that both frontal cortex and hippocampal structures are thought to play key roles in both cognition and memory function (Koechlin et al. 1999; Akhondzadeh 1999). Taken together, with our observations that 5-HT_6 receptor antagonists can enhance basal excitatory neurotransmission in both frontal cortex and hippocampus, it can be speculated that these types of compound will have therapeutic utility in the treatment of the cognitive and memory impairments associated with conditions such as schizophrenia.

In summary, we demonstrate for the first time the selective enhancement of excitatory neurotransmission, in both the frontal cortex and dorsal hippocampus, by the 5-HT_6 receptor antagonist SB-271046. These findings suggest a possible therapeutic role for 5-HT_6 receptor antagonists in the treatment of cognitive and memory dysfunction.

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ORIGINAL INVESTIGATION

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Reversal of a cholinergic-induced deficit in a rodent model of recognition memory by the selective 5-HT$_6$ receptor antagonist, Ro 04-6790

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Abstract *Rationale:* Accumulating evidence suggests a potential role for the 5-HT$_6$ receptor in cognitive function and the potential use of 5-HT$_6$ receptor antagonists in the treatment of learning and memory disorders. *Objectives:* The aim of the current study was to investigate the effect of the selective 5-HT$_6$ receptor antagonist, Ro 04-6790, on both the performance of normal adult rats and restoration of a pharmacological disruption of memory function produced by the non-selective muscarinic receptor antagonist, scopolamine, or the dopamine D$_2$ receptor antagonist, raclopride, in a rodent model of recognition memory. *Methods:* Passive, perceptually based, recognition memory was assessed using a novel object discrimination task. Following habituation to an arena, rats were presented with two identical objects during trial 1 (T$_1$) and a novel and familiar object during trial 2 (T$_2$). The time spent exploring the two objects in each trial was measured and novel object discrimination assessed in T$_2$. *Results:* In the absence of drug all rats spent an equal time exploring the two identical objects in T$_1$ but more time exploring the novel object in T$_2$. Scopolamine (but not *N*-methylscopolamine) and raclopride both produced a dose-dependent reduction in novel object discrimination whilst the 5-HT$_6$ receptor antagonist, Ro 04-6790, had no effect on discrimination when given alone but completely reversed the scopolamine- but not the raclopride-induced deficit. *Conclusion:* This study demonstrates that acute administration of Ro 04-6790 reverses a cholinergic but not a dopaminergic deficit in a rodent model of recognition memory and provides further support for a role of the 5-HT$_6$ receptor in the regulation of cognitive function.

Keywords Memory · Rats · Ro 04-6790 · 5-HT$_6$ receptor antagonists

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Introduction

The 5-HT$_6$ receptor is one of the most recent additions to the fifteen mammalian 5-HT receptors identified to date (Hoyer et al. 2002). Following the discovery of the rodent 5-HT$_6$ receptor using molecular biology (Monsma et al. 1993: Ruat et al. 1993), identification of the human analogue quickly followed (Kohen et al. 1996). These two receptor proteins comprise a linear chain of 438 (rat) and 440 (human) amino acids with a typical seven transmembrane spanning G-protein linked structure, are positively coupled to adenylyl cyclase and are 89% homologous. Research into the functional role of the receptor was initially hampered due to a lack of selective ligands and early studies made use of antisense oligonucleotides to reduce 5-HT$_6$ receptor expression (Bourson et al. 1995; Bentley et al. 1997; Yoshioka et al. 1998; Hamon et al. 1999; Otano et al. 1999). However, recently a number of selective 5-HT$_6$ receptor antagonists have been characterized (Sleight et al. 1998; Bromidge et al. 1999; Issac et al. 2000; Lee et al. 2000; Tsai et al. 2000; Slassi et al. 2000; Bös et al. 2001; Russell et al. 2001), providing more selective tools with which to probe the functional role of this receptor. To date, the receptor has been implicated in psychotic disorders (Monsma et al. 1993; Roth et al. 1994; Tsai et al. 1999; Yu et al. 1999; Pouzet et al. 2002), affective disorders (Roth et al. 1994; Yau et al. 1997; Vogt et al. 2000), anxiety (Yoshioka et al. 1998; Hamon et al. 1999; Otano et al. 1999), epilepsy (Routledge et al. 2000) and potentially the regulation of food consumption (Bentley et al. 1997, 1999b; Woolley et al.

2001), but the most compelling evidence suggests a role for the receptor in cognitive function.

Both chronic i.c.v. injection of a 5-HT_6 receptor specific antisense oligonucleotide (A.O.; Bourson et al. 1995) and acute systemic administration of 4-amino-*N*-(2,6) bis-methyl-amino-pyrimidin-4-yl-benzene sulphonamide (Ro 04-6790, Bentley et al. 1999a) produced a specific behavioural syndrome of stretching, which was blocked by atropine but not by haloperidol, suggesting that the 5-HT_6 receptor may regulate central cholinergic, but not dopaminergic, neurotransmission. Consistent with this proposal, Ro 04-6790 blocked scopolamine-induced ipsilateral rotations but had no effect on apopmorphine-induced contralateral rotations in 6-hydroxydopamine (6-OHDA) lesioned rats (Bourson et al. 1998). More recently, microdialysis studies have demonstrated elevated levels of acetylcholine in the hippocampus and frontal cortex of the conscious rat following treatment with either Ro 04-6790 (Shiraz-Southall et al. 2002) or the structural analogue Ro 65-7199 (Sleight et al. 1999). Given the well documented cholinergic link to memory function (Bartus et al. 1982), a 5-HT_6 receptor-cholinergic interaction could account for the modulation of cognition seen with 5-HT_6 receptor antagonists.

We previously demonstrated enhanced retention (but not acquisition) of a learnt platform position in normal adult rats following chronic treatment with a specific 5-HT_6 receptor-directed A.O. and the selective 5-HT_6 receptor antagonist, Ro 04-6790, after acquisition training in the Morris water maze (Bentley et al. 1997; Woolley et al. 2001). This was also seen with other 5-HT_6 receptor antagonists (5-chloro-*N*-(4-methoxy-3-piperazin-1-yl-phenyl)-3-methyl-2-benzothio-phene sulphonamide (SB-271046) and N-(2,5-dibromo-3-fluorophenyl)-4-methoxy-3-piperazin-1-yl benzene sulphonamide (SB-357134; Rogers et al. 1999; Rogers and Hagan 2001), suggesting that the 5-HT_6 receptor may regulate long-term memory in normal adult rats. More recently, Stean et al. (2002) demonstrated enhanced acquisition as measured by path length, in addition to enhanced retention of a learnt platform position in the Morris water maze following chronic administration of SB-357134 (10 mg/kg PO, twice daily, 7 days prior to training), implying a role for the 5-HT_6 receptor in both the learning and mnemonic processes involved in this spatial learning task. However, only few preliminary investigations have examined the effect of 5-HT_6 receptor antagonists on rodent models of impaired memory function, in order to determine their potential utility as therapeutic agents for the treatment of Alzheimer's disease (Rogers et al. 1999, 2000; Menses 2001).

Assessing passive perceptually based recognition memory, the novel object discrimination task takes advantage of the spontaneous preference of rodents for novelty and does not require reinforcement of behaviour by food reward. The latter aspect is of importance, since both i.c.v. injection of a 5-HT_6-receptor directed A.O. (Bentley et al. 1997) and systemic injection of a selective 5-HT_6 receptor antagonist Ro 04-6790 (Bentley et al. 1999b; Woolley et al. 2000) cause hypophagia that would confound interpretation of food motivated operant tasks (Meneses et al. 2001). Thus, using an ITI of 1 min, the novel object discrimination task used in the current study assesses short-term recognition memory. Importantly, the pharmacological validity of the rodent novel object discrimination task to predict novel compounds with potential clinical advantage has also been demonstrated since Aricept (donepezil, E2020), currently used for the symptomatic relief in Alzheimer's disease, was found to reverse an age-related deficit in this task (Ni et al. 2001).

However, to date the neural substrates of novel object discrimination have not been conclusively defined. Several groups have suggested participation of the perirhinal and entorhinal cortices (Wiig and Bilkey 1995; Ennaceur et al. 1996, 1997; Aggleton et al. 1997; Ennaceur 1998), cortical association areas (Steckler et al. 1998) and the globus pallidus (Ennaceur 1998). In contrast, the role of the hippocampus is more controversial (Steckler et al. 1998). Thus, although electrolytic lesions of the septal-hippocampal pathway have no effect on novel object discrimination (Ennaceur 1998) conflicting results have been obtained following lesions of the fimbria-fornix pathway (Ennaceur and Aggleton 1994; Ennaceur et al. 1996, 1997; Mostafa and Ennaceur 2001) and radiofrequency lesions of the hippocampus (Clark et al. 2000; Aingie et al. 2002; Mumby et al. 2002). Yet, profound hippocampal lesion by ischaemia does induce impairment (Woods and Philips 1991), which becomes apparent with intertrial intervals between 1 and 10 min (Clark et al. 2000; Mumby et al. 2002), consistent with it being involved in recognition memory but not the appreciation of novelty per se. One possible explanation for this apparent disparity is that novel object discrimination impairment is only induced following severe hippocampal lesions and intertrial intervals of greater than 1 min, as used herein.

The current study examines the effect of Ro 04-6790 on novel object discrimination, both when given alone and following the impairments induced by a muscarinic or a dopamine D_2 receptor antagonist.

Materials and methods

Animals and drug treatment

Adult male Lister hooded rats (Biomedical Services Unit, University of Nottingham) weighing 200–400 g were housed in groups of four on a 12 h light/dark cycle (lights on 7.00 a.m.) and given food and water ad libitum. Room temperature (21±1°C) and humidity (55–65%) were kept constant. Rats were randomly assigned to one of 14 treatment groups (n=10–12 per group). Pretreatment comprised scopolamine hydrobromide (0.1, 0.5 or 1 mg/kg IP), *N*-methylscopolamine (0.25 or 0.5 mg/kg IP), raclopride L-tartrate (0.1, 0.3 or 0.5 mg/kg IP), or physiological saline as the vehicle in all cases (0.154 M, 1 ml/kg IP). Twenty minutes later, rats were treated with either saline (IP), or the selective 5-HT_6 receptor antagonist Ro 04-6790 (10 or 30 mg/kg IP).

Each individual group of 10–12 rats was tested twice 7 days apart with a different pair of pretreatment/treatment conditions. On the second test, each rat was given the opposite pretreatment so that all rats received both treatments, such as saline and a chosen drug dose and thus served as their own control. Each rat was only re-

tested once in order to avoid habituation to the two objects used for all studies. Further repeated testing with alternative objects was not performed, to avoid potential habituation to the task. All experiments were performed in accordance with the UK Animals (Scientific Procedures) Act 1986, by an observer who was unaware of the treatment given.

Behavioural testing

The novel object discrimination test used in the present study was a modification of that described by Ennaceur and Delacour (1988). The apparatus comprised a clear Perspex box as the arena, measuring 39×23.5 cm with 30 cm high walls. The objects to be discriminated were plastic bottles (8 cm high×5 cm outer diameter) covered in white masking tape (familiar objects) or black and white striped masking tape (novel object). Each bottle was inverted and secured with Blue Tac through holes in the floor located 10 and 5 cm from either side and 5 cm from the end wall in opposite corners of the arena. The weight of each bottle was such that the rats could not displace it.

Twenty-four hours prior to testing, each rat was habituated to the arena for 60 min in the absence of any object. A total of 12 arenas were used and each rat was tested in the arena that it was habituated to. On test days, each group of rats received drug or saline pretreatment 20 min prior to the second drug or saline treatment and 20 min later testing began. Each rat was placed in the arena for 3 min in the absence of the objects for a second brief habituation period and then for two consecutive 3-min trial periods (T_1 and T_2, respectively). All three encounters with the arena were separated by an inter-trial interval (ITI) of 1 min, during which the rat was returned to the home cage. In the first trial (T_1), rats were exposed to two objects of identical size, shape and pattern (objects a_1 and a_2). In the second trial (T_2) one of the bottles "a_2" was replaced with a bottle of identical size and shape but with alternating horizontal black and wipe stripes ("b", the novel object), whilst a_1 was replaced with an object identical to those used in T_1 (the familiar object, "a"). During T_1 and T_2 the exploration of either object was defined as the time spent (s) sniffing, licking, chewing or touching it with the nose or within 1 cm of it with moving vibrissae and was recorded separately for each object by stopwatch. Sitting on the object was not regarded as exploratory activity (but rarely occurred). Between each session, the bottles were wiped with 20% (v/v) ethanol to remove any olfactory cues. Experiments were performed in constant light at 200 lux at floor level in the arena between 09.00 and 13.00 hours.

Statistical analysis

Within-group comparisons of time (s) spent exploring each of the two identical objects (a_1 and a_2) in trial 1 (T_1) and the novel (b) versus the familiar (a) objects in trial 2 (T_2) were analysed using the Student's paired *t*-test. The effect of treatment on overall exploratory time in T_1 and T_2 and the time spent at the novel and familiar object was compared with their appropriate controls and also analysed using the Student's paired *t*-test.

Materials

Scopolamine hydrobromide and *N*-methylscopolamine, were purchased from Sigma Chemicals (Poole, Dorset, UK), raclopride L-tartrate from RBI chemicals (Poole, Dorset, UK), and Ro 04-6790 was a gift from F. Hoffmann La-Roche (Basel, Switzerland).

Results

Pretreatment with scopolamine hydrobromide

Following treatment with saline all three groups of rats spent an equal time exploring the two identical objects (a_1 and a_2) in the first trial (T_1, Table 1), but a significantly

Table 1 Effect of increasing concentrations of the non-selective muscarinic antagonist, scopolamine hydrobromide, the quaternary amonium derivative, *N*-methylscopolamine, the dopamine D_2 receptor antagonist, raclopride and the selective 5-HT_6 receptor antagonist, Ro 04-6790, both given alone and as a combined treatment with scopolamine or raclopride, on the time spent (s, mean±SEM) exploring the two identical objects (a_1 and a_2) in trial 1. For each separate drug study, the saline data were pooled, as there was no significant difference between the groups

Treatment group	Treatment (mg/kg IP)	Number of rats	Total exploration of a_1 (s)	Total exploration of a_2 (s)
Scopolamine	Saline	35	25±2	27±2
	0.1 scopolamine	12	26±3	29±2
	0.5 scopolamine	12	18±3	19±2
	1.0 scopolamine	11	19±3	20±8
N-Methylscopolamine	Saline	24	21±2	22±1
	0.25 scopolamine	12	14±3	19±3
	0.5 scopolamine	12	16±2	16±3
Raclopride	Saline	36	24±1	21±3
	0.1 raclopride	12	24±8	23±3
	0.3 raclopride	12	16±2	17±1
	0.5 raclopride	12	18±3	16±3
Ro 04-6790	Saline	21	31±2	30±1
	10 Ro 04-6790	12	29±2	29±2
	30 Ro 04-6790	9	21±5	21±5
0.5 scopolamine+ 10 Ro 04-6790	Saline	12	17±2	21±3
	0.5 scopolamine	24	20±2	21±1
	0.5 scopolamine+10 Ro 04-6790	12	23±2	24±2
1.0 scopolamine+ 10 Ro 04-6790	Saline	12	25±3	27±4
	1.0 scopolamine	24	26±2	27±2
	1.0 scopolamine+10 Ro 04-6790	12	27±2	27±2
0.1 raclopride+ 10 Ro 04-6790	Saline	12	22±3	19±2
	0.1 raclopride	24	22±2	20±2
	0.1 raclopride+10 Ro 04-6790	12	14±2	13±1
0.3 raclopride+ 10 Ro 04-6790	Saline	12	21±1	20±1
	0.3 raclopride	24	17±1	18±1
	0.3 raclopride+10 Ro 0-46790	12	20±1	19±1

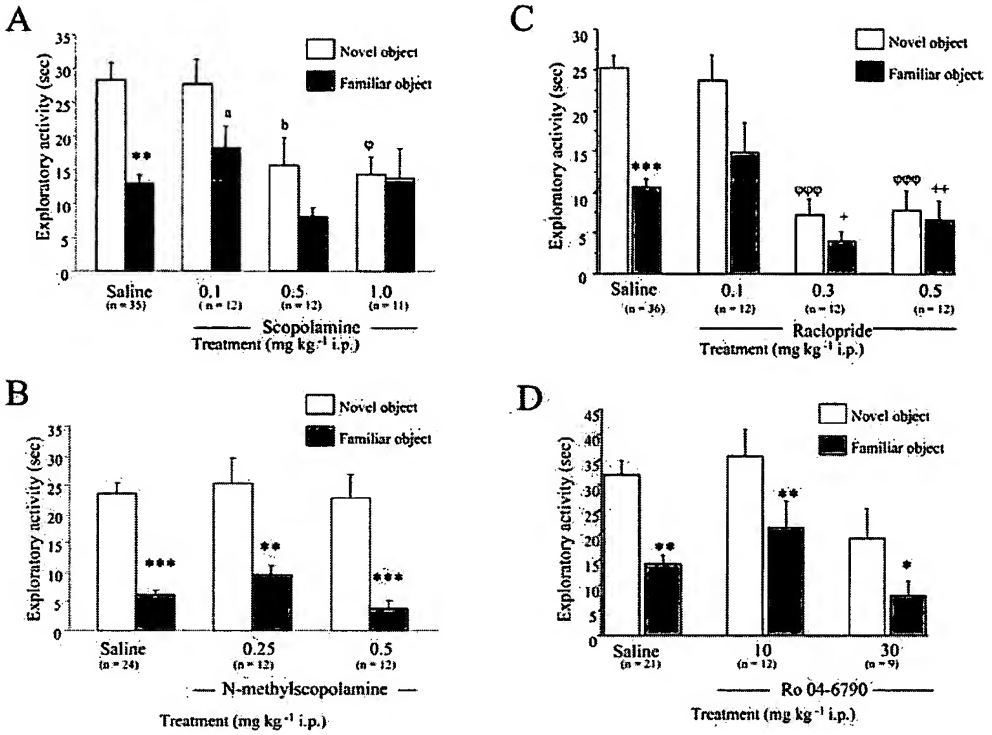

Fig. 1 Effect of increasing concentrations (as indicated, mg/kg, IP) of the non-selective muscarinic antagonist, scopolamine hydrobromide (**A**), the quaternary ammonium derivative, *N*-methylscopolamine (**B**), the dopamine D_2 receptor antagonist, raclopride (**C**) and the selective 5-HT_6 receptor antagonist, Ro 04-6790 (**D**), on the time spent (s, mean±SEM) exploring the novel versus the familiar object during trial 2. Within each separate drug study, each group of rats received a single drug dose and saline separated by an interval of 1 week and the saline data were pooled for clarity of presentation as there was no significant difference between the three groups. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, $^{a}P=0.07$ compared with time spent at the novel object in the same treatment group. $^{\phi}P<0.05$, $^{\phi\phi\phi}P<0.001$, $^{b}P=0.06$ compared with time spent exploring the novel object following saline treatment in the same group of rats. $^{+}P<0.05$, $^{++}P<0.01$ compared with time spent at the familiar object following saline treatment in the same group of rats

greater time exploring the novel (b) versus the familiar object (a) in the second trial (T_2, $P<0.01$, in each case, Fig. 1A), showing that they were able to discriminate the novel object during the choice trial. Following treatment with scopolamine (0.1–1 mg/kg IP), all rats also spent an equal time exploring the two identical objects (a_1 and a_2) in T_1 (Table 1). However, scopolamine (0.1, 0.5, and 1 mg/kg) caused a dose-dependent impairment of novel object discrimination in T_2, such that rats spent equivalent times exploring the novel and the familiar objects (Fig. 1A). This effect was most pronounced with the two higher doses of scopolamine, since the difference in time spent exploring the novel versus the familiar object following pretreatment with the lower dose of (0.1 mg/kg) scopolamine just missed significance ($P=0.07$). Notably, scopolamine (0.5 and 1.0 mg mg/kg) tended to reduce the overall exploratory activity in T_2, although when compared with saline treatment in the same rats, this did not reach significance. However, further analysis shows that scopolamine (0.5 and 1.0 mg/kg) selectively reduced the time spent at the novel ($P=0.06$ for 0.5 mg/kg and $P<0.05$ for 1.0 mg/kg scopolamine) and not the familiar object when compared with saline pretreatment in the same rats (Fig. 1A). Thus 0.5 and 1.0 mg/kg were the doses of scopolamine chosen to examine the effect of Ro 04-6790 on a scopolamine-induced deficit in novel object discrimination.

Pretreatment with *N*-methylscopolamine

Following treatment with saline rats spent an equal time exploring the two identical objects during T_1 and significantly longer exploring the novel versus the familiar object during the choice trial ($P<0.001$, Fig. 1B). Similarly, following treatment with N-methylscopolamine (both 0.25 and 0.5 mg/kg), all rats spent an equal time exploring the two identical objects during T_1 (Table 1). In contrast to scopolamine (above), treatment with *N*-methylscopolamine (0.25 or 0.5 mg/kg), which does not penetrate into the CNS, had no effect on the ability of the rats to discriminate the novel object in T_2. Thus all rats given *N*-methylscopolamine, irrespective of dose, spent a significantly longer time exploring the novel versus the familiar object ($P<0.01$ for 0.25 and $P<0.001$ for 0.5 mg/kg *N*-methylscopolamine, Fig. 1B) Notably, *N*-methylscopolamine also had no effect on total object exploration time in either trial (Table 2).

Table 2 Effect of scopolamine, *N*-methylscopolamine, raclopride and the 5-HT_6 antagonist, Ro 04-6790 at the doses indicated (mg/kg IP) on total object exploration time (s, mean±SEM) in trial 1 (T_1) and trial 2 (T_2) (i.e. the total time spent at the two familiar objects during T_1 and the novel and familiar object during T_2). For each separate drug study the saline data were pooled, as there was no significant difference between the groups

Dose-response curve	Treatment (mg/kg IP)	Number of rats	Total object exploration (s) in T_1	Total object exploration (s) in T_2
Scopolamine	Saline	35	52±3	41±3
	0.1 scopolamine	12	54±4	46±4
	0.5 scopolamine	12	36±4	24±5
	1.0 scopolamine	11	40±5	28±7
N-Methylscopolamine	Saline	24	43±3	29±2
	0.25 scopolamine	12	33±5	37±6
	0.5 scopolamine	12	33±5	23±4
Raclopride	Saline	36	46 ±2	36±2
	0.1 raclopride	12	46±5	39±5
	0.3 raclopride	12	33±3*	11±3**
	0.5 raclopride	12	34±6*	15±5***
Ro 04-6790	Saline	21	62±3	54±4
	10 Ro 04-6790	12	58±4	49±9
	30 Ro 04-6790	9	50±8	39±6

*$P<0.05$, **$P<0.01$ and ***$P<0.001$ compared with saline pretreatment in the same rats

Pretreatment with raclopride

As seen previously, following treatment with saline, rats spent an equal time exploring the two identical objects in the first trial (T_1, Table 1) but a significantly greater time ($P<0.001$) exploring the novel versus the familiar object in the second trial (Fig. 1C). In contrast, treatment with raclopride, irrespective of dose, impaired novel object discrimination in T_2 (Fig. 1C) without altering the pattern of exploration in T_1 (Table 1). Notably, pretreatment with raclopride (0.3 mg/kg and 0.5 mg/kg) also reduced the total exploratory activity in T_1 when compared with that of saline pretreatment ($P<0.05$ for both groups, Table 2). This effect was also seen in T_2 ($P<0.01$ for 0.3 mg/kg and $P<0.001$ for 0.5 mg/kg, Table 2), and further analysis showed that raclopride reduced both the time spent exploring the novel ($P<0.001$ in both cases) and the familiar object ($P<0.05$ for 0.3 mg/kg and $P<0.01$ for 0.5 mg/kg, Fig. 1C) in T_2, suggesting that these doses caused a non-selective reduction in exploration rather than a selective attenuation of working memory. Thus the lower doses (0.1 and 0.3 mg/kg) of raclopride were chosen to examine the effect of Ro 04-6790 on a raclopride-induced deficit in novel object discrimination.

Treatment with Ro 04-6790

Following treatment with saline, both groups of rats spent an equivalent time exploring the two familiar objects during T_1 (Table 1) but significantly longer exploring the novel versus the familiar object during T_2 ($P<0.01$, Fig. 1D). Treatment with Ro 04-6790 had no effect on novel object discrimination, such that even following the highest dose (30 mg/kg) of this 5-HT_6 antagonist, rats spent an equal time exploring the two identical objects in T_1 (Table 1) and significantly longer exploring the novel versus the familiar object in T_2 ($P<0.01$ for 10 mg/kg Ro 04-6790 and $P<0.05$ for 30 mg/kg Ro 04-6790, Fig. 1D). Thus, Ro 04-6790 had no effect on the ability of the rats to discriminate the novel object in T_2. In contrast to the effect of the lowest dose of Ro 04-6790, treatment with 30 mg/kg tended to reduce total object exploration time during T_2 (Table 2), but this did not reach significance.

Ro 04-6790 reverses a scopolamine- but not a raclopride-induced deficit in novel object discrimination

As expected from the dose-response study, pretreatment with both 0.5 and 1 mg/kg scopolamine impaired the ability of the rats to discriminate the novel from the familiar object in T_2 (Fig. 2A, B), without altering the pattern of exploration in T_1 (Table 1), such that in both cases rats spent an equal time exploring the novel and familiar objects in the choice trial. However, in both cases, treatment with Ro 04-6790 (10 mg/kg IP) completely reversed the scopolamine-induced deficit in novel object discrimination (Fig. 2A, B) such that rats receiving combined treatment of scopolamine (0.5 or 1.0 mg/kg) and Ro 04-6790 spent a significantly longer time exploring the novel versus the familiar object in T_2 ($P<0.01$ for 0.5 mg/kg scopolamine + Ro 04-6790 and $P<0.001$ for 1.0 mg/kg scopolamine + Ro 04-6790).

Notably, when given in combination with scopolamine (0.5 mg/kg) in T_2, Ro 04-6790 (10 mg/kg) increased the total exploratory activity compared with that in the same rats receiving scopolamine alone ($P<0.05$, Table 3). Although it failed to reach significance, Ro 04-6790 produced a similar increase in the response in rats receiving the higher dose of scopolamine (1.0 mg/kg, Table 3). Further analysis showed that in both cases, this effect was accounted for by a significant increase in time spent exploring the novel object during T_2 ($P<0.05$ in both cases, Fig. 2A and 2B) following combined treatment with scopolamine and Ro 04-6790, consistent with the rats selectively redirecting their exploration towards the novel object in the choice trial.

As expected from the dose-response curve (Fig. 1C), pretreatment with raclopride (0.3 mg/kg IP) abolished novel object discrimination in T_2 (Fig. 3B) without altering the pattern of object exploration in T_1 (Table 1).

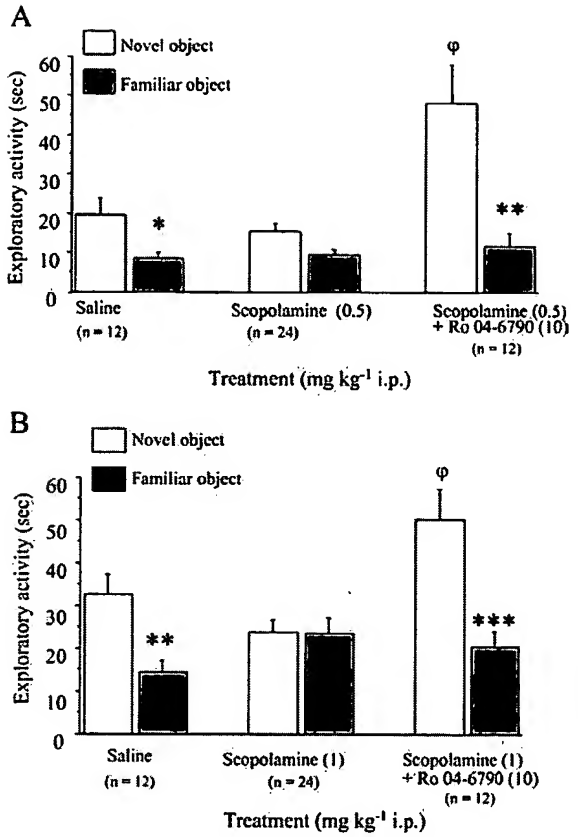

Fig. 2 Effect of combined treatment with Ro 04-6790 (10 mg/kg IP) and 0.5 mg/kg IP scopolamine (**A**) or 1 mg/kg IP scopolamine (**B**) on the time spent (s, mean±SEM) exploring the novel versus the familiar object during trial 2. Within each of the studies the two groups of rats received either scopolamine and saline or scopolamine and Ro 04-6790 separated an interval of 1 week. For clarity of presentation the data from the two groups were pooled for each drug. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ compared with time spent at the novel object in the same treatment group. $^{\phi}P<0.05$ compared with time spent exploring the novel object following treatment with scopolamine alone in the same rats

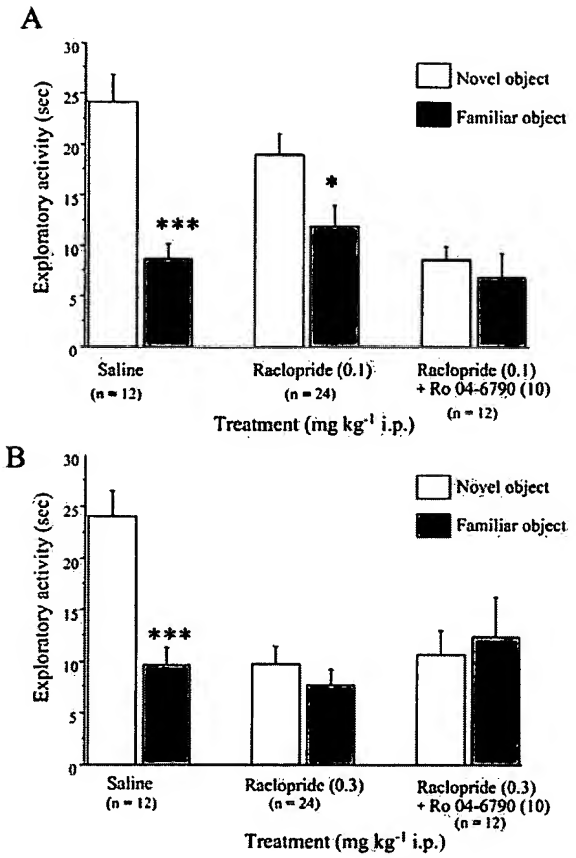

Fig. 3 Effect of combined treatment with Ro 04-6790 (10 mg/kg IP) and 0.1 mg/kg IP raclopride (**A**) or 0.3 mg/kg IP raclopride (**B**) on the time spent (s, mean±SEM) exploring the novel versus the familiar object during trial 2. Within each of the studies each group of rats received either raclopride and or raclopride and Ro 04-6790 separated by an interval of 1 week. For clarity of presentation, the data from the two groups were pooled for each drug. $^{*}P<0.05$, $^{***}P<0.001$ compared with time spent at the novel object in the same treatment group

Table 3 Effect of scopolamine (0.5 and 1.0 mg/kg IP) or raclopride (0.1 or 0.3 mg/kg IP) either given alone or as a combined treatment with Ro 04-6790 (10 mg/kg IP) on the total object exploration time [total time spent at both objects (s, mean±SEM)] in trial 1 (T_1) and trial 2 (T_2)

Treatment group	Treatment (mg/kg IP)	Number of rats	Total object exploration (s) in T_1	Total object exploration (s) in T_2
0.5 scopolamine+10 Ro 04-6790	Saline	12	38±4	28±4
	0.5 scopolamine	24	41±3	25±3
	0.5 scopolamine+10 Ro 04-6790	12	47±3	60±9*
1.0 scopolamine+10 Ro 04-6790	Saline	12	51±7	47±7
	1.0 scopolamine	24	50±3	47±6
	1.0 scopolamine+10 Ro 04-6790	12	54±4	71±9
0.1 raclopride+10 Ro 04-6790	Saline	12	47±5	33±3
	0.1 raclopride	24	42±3	31±3
	0.1 raclopride+10 Ro 04-6790	12	26±2	15±3
0.3 raclopride+10 Ro 04-6790	Saline	12	41±2	34±3
	0.3 raclopride	24	36±2	18±3
	0.3 raclopride+10 Ro 04-6790	12	39±2	23±6

*$P<0.05$, compared with scopolamine treatment alone in the same rats

Similarly, rats receiving combined treatment with raclopride (0.3 mg/kg) and Ro 04-6790 (10 mg/kg) were also unable to discriminate the novel from the familiar object and spent an equal time at the two objects in T_2 (Fig. 3B). Conversely pretreatment with the lower dose (0.1 mg/kg) of raclopride did not impair novel object discrimination but combined treatment of 0.1 raclopride and Ro 04-6790 further attenuated, and led to a significant impairment of, novel object discrimination (Fig. 3A). Thus, in contrast to the results seen with scopolamine, Ro 04-6790 did not reverse the raclopride-induced deficit in novel object discrimination.

Discussion

The aim of the present study was to investigate the effect of the selective 5-HT_6 receptor antagonist, Ro 04-6790, both when given alone and following a pharmacologically induced deficit in a rodent model of short term recognition memory, the novel object discrimination task. Acute systemic injection of Ro 04-6790 had no effect on novel object discrimination when given alone but it totally reversed the deficit induced following blockade of muscarinic but not dopamine D_2 receptors.

The centrally active, non-selective muscarinic receptor antagonist, scopolamine, has been widely used to demonstrate the cholinergic involvement in memory and cognition in rodents. Peripheral administration of scopolamine produces deficits in a variety of spatial tests such as the delayed matching to position (Dunnett 1985) and the Morris water maze (Harder et al. 1996). In addition, deficits in non-spatial working memory tests such as spontaneous alternation (Pontecorvo et al. 1991), novel object discrimination (Ennaceur and Meliani 1992) and continuous non-matching tasks (Wan et al. 1997) occur following muscarinic antagonist administration. Consistent with these findings, pretreatment with scopolamine at doses equivalent to those used in the aforementioned studies (Dunnett 1985; Ennaceur and Meliani 1992) also produced impaired novel object discrimination in the current study. In contrast, acute, systemic injection of the quaternary ammonium derivative *N*-methylscopolamine, that does not penetrate the blood-brain barrier and therefore has no CNS activity, had no effect on novel object discrimination, consistent with previous reports (Dunnett 1985; Ennaceur and Meliani 1992). Taken together, this suggests that the deficit in novel object discrimination seen with scopolamine is mediated via central muscarinic receptor blockade and not due to a parasympatholytic effect on visual acuity.

In accordance with previous studies demonstrating attenuation of working memory in both spatial (Wilkerson and Levin 1999; Umegaki et al. 2001) and non-spatial tasks (Didriksen 1995), the selective dopamine D_2 receptor antagonist raclopride also caused a dose-dependent impairment of novel object discrimination. This is consistent with the proposed role of dopamine D_2 receptors in the regulation of memory (Noyce et al. 1993). However, the deficit in novel object discrimination seen following treatment with the highest dose (0.3 mg/kg) of raclopride was also accompanied by a reduction in total object exploration, in both trials 1 and 2. Thus, in the current study it seems that the deficit in novel object discrimination seen with raclopride was a consequence of the characteristic hypolocomotor effect of dopamine D_2 receptor antagonists (Jackson and Westlind-Danielsson 1994; Feldman et al. 1997), rather than a selective attenuation of recognition memory.

The selective 5-HT_6 receptor antagonist Ro 04-6790 has been shown to have over 100-fold selectivity for the 5-HT_6 receptor compared with 24 other G-protein coupled receptors, including all muscarinic, dopamine and eight other 5-HT receptors (Sleight et al. 1998). When given alone, Ro 04-6790, had no effect on the ability of the rats to perform discrimination of a novel object. However, it completely reversed the deficit in novel object discrimination produced by scopolamine, suggesting that blockade of 5-HT_6 receptors increases cholinergic function sufficiently to overcome central muscarinic receptor blockade, probably by increasing acetylcholine release (Sleight et al. 1999; Shirizai-Southall et al. 2002). Conversely, Ro 04-6790 did not reverse the dopamine D_2 receptor antagonist-induced deficit in the same task, consistent with previous data indicating that the 5-HT_6 receptor does not modulate central dopaminergic neurotransmission (Bourson et al. 1995, 1998; Bentley et al. 1999a; Dawson et al. 2000, 2001). Since D_2 dopamine receptor blockade following treatment with raclopride in the current study produces a non-selective reduction in exploratory behaviour, the fact that Ro 04-6790 did not reverse this non-specific behavioural disturbance, but did reverse a selective reduction in novel object discrimination following scopolamine, further supports a role for the 5-HT_6 receptor in memory processes.

Consistent with the data in the current study, several lines of evidence suggest that 5-HT_6 receptors regulate central cholinergic neurotransmission. For instance, both 5-HT_6-directed A.O. (Bourson et al. 1995) and Ro 04-6790 (Sleight et al. 1998) produced yawning, stretching and chewing that was antagonised by the muscarinic receptor antagonists atropine and scopolamine (Bourson et al. 1995; Bentley et al. 1999a). Routledge et al. (1999) also demonstrated that another 5-HT_6 antagonist, SB-271046 (Bromidge et al. 1999), enhanced physostigmine-induced yawning. Since the behavioural responses were seen after treatment with a 5-HT_6 receptor-directed A.O. or a selective antagonist, it has been proposed that this receptor receives tonic serotonergic input or possesses constitutive activity (Bourson et al. 1995). Furthermore, after unilateral 6-OHDA lesions of the medial forebrain bundle, both atropine- and scopolamine-induced ipsilateral rotations, but not dopamine-induced contralateral rotations, were inhibited by Ro 04-6790 (Bourson et al. 1998). In agreement with a 5-HT_6 receptor-cholinergic interaction in the regulation of cognition, Meneses (2001) demonstrated that Ro 04-6790 (5 mg/kg IP) reversed a scopolamine-induced deficit in learning consolidation in

an autoshaping paradigm. Furthermore, two preliminary intracerebral microdialysis reports suggest that Ro 04-6790 and the structural analogue Ro 65-7199, increase cortical and hippocampal extracellular acetylcholine levels (Sleight et al. 1999; Shirazi-Southall et al. 2002, respectively) and reverse scopolamine-induced deficits in the Morris water maze and passive avoidance tests (Sleight et al. 1999; Bös et al. 2001).

Whilst the present study demonstrates a 5-HT_6 receptor-cholinergic interaction, the way in which 5-HT_6 receptor blockade increases acetylcholine release is currently unknown. By using an N-terminal directed specific antibody, we previously characterised the distribution of 5-HT_6-like immunoreactivity (5-HT_6-LI) in the rat brain (Woolley et al. 2000). The overall pattern matched that reported using a C-terminal directed antibody (Gérard et al. 1997) and the selective radioligand [^{125}I] 4-iodo-N-[4-methoxy-3-(4-methylpiperazin-1-yl)phenyl]benzenesulfonamide (SB-258585, Roberts et al. 2002), demonstrating abundant 5-HT_6 receptor protein in several of the neural areas implicated in the novel object discrimination task, including the cortex and hippocampus (Steckler et al. 1998; Clark et al. 2000; Mumby et al. 2002). Furthermore, by using dual labelled immunohistochemistry, very low levels of co-existence were found between the 5-HT_6 receptor and choline acetyltransferase (ChAT)-LI (Woolley et al. 2000) limited to few discrete brain regions, including the medial septal nuclei (17% of 5-HT_6 positive neurones) caudate nucleus (19%), nucleus accumbens (16%) and some areas of the cortex (5–8% in the cingulate, frontal and parietal corticies). Taken together, these data suggest that whilst a direct 5-HT_6 receptor activation of cholinergic neurones may occur in these areas, in some regions the predominant form of interaction may not be direct. Conversely, abundant and extensive co-existence was seen between 5-HT_6-LI and GAD_{67}-LI, a marker of GABAergic neurones in 29 out of the 42 brain regions examined (Woolley et al. 2000). Such co-existence is in accordance with previous evidence of 5-HT_6 receptor-GABA co-existence in the striatum (Ward and Dorsa 1996; Gérard et al. 1997), and suggests that this may be an important method of interaction. Therefore, the 5-HT_6 receptor-regulation of cholinergic neurotransmission may occur via inhibition of GABA and thence disinhibition of acetylcholine release, as previously described for the 5-HT_3 receptor (Ramirez et al. 1996; Diez-Ariza et al. 1998).

Interestingly, Dawson et al. (2001) recently demonstrated that the 5-HT_6 receptor antagonist, SB-271046, caused a tetrodotoxin-sensitive increase in extracellular glutamate release by microdialysis in the frontal cortex and dorsal hippocampus (but not in the striatum or nucleus accumbens) of conscious adult rats. Furthermore, Meneses (2001) demonstrated that Ro 04-6790 partially reversed an impairment in learning consolidation produced by the NMDA receptor antagonist, dizocilpine, consistent with the idea that 5-HT_6 receptor-modulation of glutamate may also contribute to the effect of 5-HT_6 receptor antagonists on memory processing. Interestingly, serotonergic median raphé afferents preferentially innervate inhibitory GABAergic interneurones in the hippocampus and dentate gyrus (Freund et al. 1990; Freund 1992) and thus is consistent with the proposal that 5-HT_6 receptor regulation of glutamate may occur indirectly via inhibition of GABA in this area. Indeed, Dawson et al. (2001) showed that cholinergic-regulation of glutamate release in this area is unlikely, since atropine had no effect on SB-271046-induced extracellular glutamate release. However, the possibility that cholinergic neurotransmission increases as a result of elevated glutamatergic neurotransmission has not been investigated in this region and cannot be ruled out, since cholinergic interneurones are present, albeit at low levels (Vizi and Kiss 1998), and such an interaction has been demonstrated in the rat striatum (Consolo et al. 1996).

In summary, the current study demonstrates that acute systemic administration of Ro 04-6790 selectively reverses a cholinergic-induced deficit in a rodent model of short term recognition memory. Whilst the underlying mechanism of action remains to be elucidated the current findings add further support for a role of the 5-HT_6 receptor in the regulation of memory processes.

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5-HT$_6$ RECEPTORS AS EMERGING TARGETS FOR DRUG DISCOVERY

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■ **Abstract** 5-ht$_6$ receptors are the latest serotonin receptors to be identified by molecular cloning. Their high affinity for a wide range of drugs used in psychiatry, coupled with their intriguing distribution in the brain, has stimulated significant interest. Antisense oligonucleotides, antipeptide antibodies, selective radioligands, knockout mice, and selective antagonists of the 5-ht$_6$ receptor have recently become available. Surprisingly, 5-ht$_6$ receptors appear to regulate cholinergic neurotransmission in the brain, rather than the expected interaction as modulators of dopaminergic transmission. This interaction predicts a possible role for 5-ht$_6$ receptor antagonists in the treatment of learning and memory disorders. Furthermore, polymorphisms in the sequence of the 5-ht$_6$ receptor gene may provide a genetic tool to further our understanding of the differential responses of patients to antipsychotic medications.

INTRODUCTION

The multiplicity of actions of serotonin has been known for decades (1). Although the pharmacology of these responses suggested the likelihood of receptor subtypes (2, 3), the first identification of a discreet molecular species of known (deduced) amino acid composition came only a decade ago (4). This discovery presaged an explosion in the number of genetically identified receptors for serotonin (5). Attendant to the relatively fast pace of molecular discovery has been the slower pace of receptor characterization using nucleotide probes, radioligands, known chemical entities, and biochemical functional assays. More recently, the development of newly created probes such as selective antibodies, selective agonists and antagonists, knockout animals, and genetic linkages studies has been essential to further our understanding of the functions of individual serotonin receptor subtypes. Although our knowledge is far from complete, there is now substantial progress in the elucidation of functions of the most recently discovered serotonin receptor, 5-ht$_6$, and its possible therapeutic roles.

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MOLECULAR BIOLOGY OF 5-HT$_6$ RECEPTORS

The first cloning of the rat 5-ht$_6$ receptor reported a sequence predicted to encode a protein of 436 amino acids (6, 7). This sequence has been re-evaluated, and the receptor is now deduced to form a protein of 438 amino acids. The human homologue contains 440 amino acids (8). The human and rat amino acid sequences are 89% identical. Structural elements of this receptor sequence include a relatively short third intracellular loop (50 amino acids, rat; 57 amino acids, human) and a long carboxyl tail (120 amino acids), both of which are common to some other GPCRs that couple to adenylate cyclase stimulation (9). The 5-ht$_6$ receptor has a single glycosylation site in the amino terminus and multiple potential phosphorylation sites for protein kinase C in the cytoplasmic domains. The sequence also contains a leucine zipper motif in transmembrane (TM) III (7). The sequence has the highest amino acid identity (37%) to the *Drosophila melanogaster* cyclase stimulatory serotonin receptor (10) and the histamine H_2 receptor (11); lower identities are observed with 5-ht$_5$ and 5-HT$_7$ receptors. The rat and human sequences each contain two introns, one in the third intracellular loop and the other in the third extracellular loop (8). The intron in the third cytoplasmic loop appears at the same location as that described for the 5-ht$_{5a}$ and 5-ht$_{5b}$ receptors, as well as the dopamine D_2 and D_3 receptors (8). No additional subtypes have been cloned, and no functional splice variants have been identified. However, a truncated variant of the 5-ht$_6$ receptor, first noted by Monsma et al (6), was presumed to occur as a mis-splicing event and delete the seventh TM VII domain (12). Subsequently, Olsen and coworkers (13) showed that the human 5-ht$_6$ gene could give rise to an alternate splicing of the first intron, which produced a truncated variant of the receptor containing the amino terminus through TM IV. This variant is transcribed, and the transcripts are expressed in a limited subset of the brain regions (substantia nigra and caudate) occupied by mRNA for the full-length receptor.

The gene for the human 5-ht$_6$ receptor maps to chromosome region 1p35–p36, thus overlapping with the gene locus for the 5-HT$_{1D\alpha}$ receptor (8). The 5-ht$_6$ receptor sequence contains a Rsa1 restriction fragment–length polymorphism in the first extracellular loop (C267T).

MOLECULAR PHARMACOLOGY OF 5-HT$_6$ RECEPTORS

The 5-ht$_6$ receptor can be radiolabeled with [^{125}I]lysergic acid diethylamide (LSD) and couples to the stimulation of adenylate cyclase (6, 7). The distinctive properties of the pharmacology of the cloned rat 5-ht$_6$ receptor are its high affinity for a series of antipsychotic compounds, including clozapine and loxapine, as well as affinity for a number of tricyclic antidepressants such as amoxipine, clomipramine, and amitryptyline (6, 14, 15). A new potential antipsychotic compound, BIMG 80, also has moderate affinity for the 5-ht$_6$ receptor (16). Analysis of

binding studies using [^{125}I] LSD as a radioligand gives the rank order of affinities: methiothepin > 5-MeOT > 5-HT > tryptamine > 5-CT > sumatriptan >> 8-OH-DPAT. The affinity of 5-HT to the 5-ht$_6$ receptor is relatively low compared with other serotonin receptors. A similar receptor profile has been reported in N8TG2 cells, a neuroblastoma line. It displays a rank order of agonist potency in both radioligand binding and cAMP assays: 5-MeOT > 5-HT > tryptamine > 2-methyl tryptamine > 5-CT > α-methyl-5-HT (17). Responses to 5-HT in this cell line are antagonized by clozapine. Affinities of compounds for the human cloned 5-ht$_6$ receptor are equivalent to those determined for the rat, with the exception of four compounds: methiothepin (4-fold higher affinity for the human receptor), metergoline, and the atypical antipsychotics, tiopyrone and amperozide (>10-fold higher affinity for the rat receptor). Clozapine is a high-affinity antagonist at both human and rat 5-ht$_6$ receptors. The rank order of binding affinities for antagonists is methiothepin > clozapine = olanzapine > ritanserin >> risperidone. A nonconserved amino acid substitution of threonine (rat) for leucine (human) in TM III may contribute to the differences in binding affinities observed between the species homologues.

The primary signal transduction pathway of the 5-ht$_6$ receptor is the stimulation of adenylate cyclase (AC) activity. The rank order of both agonist and antagonist potencies, as well as their quantitative values, determined from AC stimulation matches closely with those determined in parallel using radioligand binding (15, 18). There are multiple isoforms of AC; for example, AC5 is a G_s-sensitive AC. It is highly localized in the striatum and nucleus accumbens, two major areas of 5-ht$_6$ localization. In contrast, AC1 and AC8 are calmodulin-stimulated ACs and are not activated by G_s proteins in vivo. AC1 and AC8 are neural-specific cyclases. AC1 is expressed in hippocampus, and AC8 is expressed in hippocampus and hypothalamus. The 5-ht$_6$ receptor, expressed in HEK 293 cells, interacts specifically with AC5 but not with AC1 or AC8 (19).

A limited number of mutations have been made experimentally to probe the binding pocket of the 5-ht$_6$ receptor. In TM V of many monoamine receptors, there are two "conserved" serine residues that are responsible for hydrogen bonding of hydroxyl groups of the cognate neurotransmitter (20, 21). In the many 5-HT receptors, the second serine is replaced by an alanine, and this replacement affects the binding of compounds such as N-1–substituted ergolines and tryptamines (22, 23). In the 5-ht$_6$ receptor, a threonine, rather than the "expected" alanine, occupies this position. Mutation of this residue to alanine (T196A) results in a decrease in the affinity of the mutant for LSD, 5-HT, and other N-1 unsubstituted ergolines (24). The magnitude of this change is consistent with a disruption of a hydrogen bond. In contrast, the N-1–methylated ergolines showed unchanged or enhanced affinity. Mutations were also made in TM III and TM VI (25, 26). In TM III, mutation of the conserved aspartic acid to asparagine (D106N) resulted in a loss of [^{3}H]LSD binding, although AC stimulation could still be elicited with both LSD and 5-HT. The potencies, however, were right shifted by 500-fold for LSD and 3600-fold for 5-HT (25). This change in affinity is consis-

tent with the loss of a charge-charge interaction. In contrast, mutation of the conserved tryptophan one helical turn upstream of this mutation (W102F) resulted in only a small (two- to sixfold) reduction of affinity for most test compounds. Finally, two adjacent residues near the distal end of TM VI were probed (A287L, N288S) as doubled mutants (25). This mutant displayed an elevated affinity for tryptamine derivatives with large substitutions on the 5′ position, as well as for ergopeptine ligands with large substituents on the 2′ position, possibly consistent with formation of a new hydrogen bond to Ser288. Studies such as these will aid molecular modeling approaches used in the design of selective ligands for this receptor.

REGULATION OF 5-HT$_6$ RECEPTORS

Since 5-ht$_6$ receptors may be important mediators of some of the beneficial actions of psychiatric drugs, it would be interesting to know if 5-ht$_6$ receptors act as autoreceptors. To investigate this possibility, Gerard et al (27) evaluated the impact of selective lesioning of the serotonergic system on the distribution of 5-ht$_6$ receptors by using 5,7-dihydroxytryptamine. Three weeks after administration of the toxin by microinfusion, only 10% of the serotonin transporter, a marker of serotoninergic neurons, remained in the anterior raphe region. In contrast, 5-ht$_6$ mRNA levels were unchanged. This observation indicates that 5-ht$_6$ receptors are not located on serotoninergic neurons, and therefore are not autoreceptors. In addition, the postsynaptic target cells of the serotoninergic projections do not up- or down-regulate their 5-ht$_6$ mRNA levels in response to the lesion, at the time point examined.

Glucocorticoids are known to affect serotoninergic systems and to be related to depression (28). Blockade of glucocorticoid synthesis, with metyrapone and aminogluthethimide, increases 5-ht$_6$ mRNA levels in the CA1 regions of the hippocampus (29). This effect can be partially reversed with corticosterone replacement. Metyrapone and aminogluthethimide have both been used in resistant depression, which has led to speculation that increases in receptor number with these treatments may enhance the effect of antidepressant ligand (29).

The developmental expression of mRNA for the 5-ht$_6$ receptor has been studied using RT-PCR. Expression of these transcripts first appeared on embryonic day 12 (E12), coincident with the appearance of the first serotonergic cell bodies of brain neurons, suggesting a possible role of the 5-ht$_6$ receptor in growth factor properties of 5-HT (30). Expression increased through postnatal day 15 and then stabilized through adult at the same level.

Selective Agonists and Antagonists for 5-ht$_6$ Receptors

At present, there are no fully selective agonists. However, a careful structure-affinity analysis of 5-HT derivatives has been presented (31). The most selective agonist is 2-methyl-5-HT. Modifications on the 5 position indicate that the

hydroxyl group is relatively unimportant for 5-HT binding. The conformation of the side chain that the 5-ht$_6$ receptor prefers for binding is that adopted by ergolines. An intact indole nucleus is favored for binding. Secondary and tertiary amines are preferred over the primary amine or the quaternary amine, whereas large dialkyl substitutions reduce affinity.

It was previously shown that many known antidepressants and antipsychotics are antagonists of the 5-ht$_6$ receptor (14). However, none was selective for this receptor. They typically have affinity for dopamine receptors, other 5-HT receptors, monoamine oxidase, and many other sites. Great strides have been made recently in the development of selective antagonists of the 5-ht$_6$ receptor.

The first reported 5-ht$_6$ antagonists were Ro-04–6790 [4-amino-N-(2,6 bis-methylamino-pyrimidin-4-yl)-benzene sulphonamide] and Ro-63–0563 [4-amino-N-(2,6 bis-methylamino- pyridin-4-yl)-benzene sulphonamide] (32). They are both relatively high-affinity (pK_i = 7.3 and 7.9, respectively), selective competitive antagonists (pA_2 = 6.75 and 7.10), as evaluated in transfected cells. There are no significant differences in their affinities for rat compared with human 5-ht$_6$ receptors. Ro-04–6790 can be administered i.p. and is CNS penetrant. Ro-63–0563 can be administered i.v. and is also CNS penetrant. The preferred compound for in vivo use is Ro-04–6790, although neither compound achieves high brain levels.

These structures were evaluated for use as radioligands. [^{3}H]Ro 63–0563 was synthesized and had a specific activity of 29 Ci/mmol (33). It was used in membrane binding studies in rat striatal membranes. The measured dissociation constant was 11.7 nM and the B_{max} was 175 fmol/mg protein. However, poor levels of specific binding were observed (10–30%). In transfected cells, the ligand had a dissociation constant of 5 nM for human 5-ht$_6$ and 6.8 nM for rat 5-ht$_6$, and a better specific binding level was obtained (70%). The pharmacological profile of the 5-ht$_6$ receptor as determined using this radioligand was not significantly different from that measured with [^{3}H]LSD as a radioligand. The high nonspecific binding in native tissues limits its use as a radioligand for autoradiographic studies.

The next selective antagonist was SB-271046 (34). The initial hit from a high throughput screen was 4-bromo-N-[4-methoxy-3-(4-methylpiperazin-1-ylphenyl] benezenesulfonamide. It had a pK_i of 8.3 nM and was 50-fold selective over other binding sites. This compound was moderately brain penetrant but was rapidly cleared from the blood and therefore had low oral bioavailability. A series of bisaryl sulfonamides was prepared and evaluated. The 5-chloro-3-methylbenzothiophene derivative had a subnanomolar affinity but was metabolized by N-dealkylation in the rat to yield the corresponding NH-piperazine. Synthesis of this metabolite, which was detected at a high level in blood, resulted in a 5-ht$_6$ antagonist with high affinity (pK_i = 8.9 nM) and potency (pA_2 = 8.7 nM) and excellent selectivity. The compound was found to be moderately CNS penetrant (10%) and to have a low blood clearance, good half-life (4.8 h in rat), and 80% oral bioavailability. As such, 5-chloro-N-(4-methoxy-3-piperazin-1-yl-phenyl)-3-

methyl-2-benzothiophenesulfonamide (SB-271046) is a significant new tool for the study of 5-ht_6 receptor function.

In addition, another compound from the series {5-iodo-N-[4-methoxy-3-(4-methylpiperazin-1-yl-phenyl]benezenesulfonamide} has been radioiodinated to form [^{125}I]SB-258585 and evaluated for use as a radioligand (35). [^{125}I]SB-258585 had a specific activity of 2000 Ci/mmol. In binding assays on membranes derived from human 5-ht_6–transfected cells, a K_d = 0.8 nM was determined and 95% specific binding was obtained. Subsequent studies in native tissue homogenates (36) indicate that there is high specific binding (60–68%) in rat and pig striatal membranes and in human caudate-putamen membranes. The K_d in rat and porcine tissues was 2.8 nM with a B_{max} of 180 fmol/mg protein. In human caudate the K_d was 1.3 nM with a B_{max} of 215 fmol/mg protein. The rank order of affinities for a discriminating set of ligands was comparable to that previously determined for the cloned receptors and also by using alternate radioligands, thereby validating this as a new tool for the study of 5-ht_6 receptors. In addition, this ligand is useful for autoradiographic mapping studies (37) (see below).

IN VITRO STUDIES

Localization of mRNA for the 5-ht_6 Receptor

The distribution of mRNA encoding the 5-ht_6 receptor has been determined by Northern blot analysis in the rat brain and peripheral tissues (6). The highest expression was detected in the striatum with lower-density signals detected in the amygdala, cerebral cortex, and olfactory tubercle. mRNA was undetectable in cerebellum, hippocampus, hypothalamus, medulla, olfactory bulb, pituitary, retina, thalamus, and a number of peripheral tissues (heart, lung, kidney, liver, spleen, pancreas, skeletal muscle, smooth muscle, stomach, ovary, prostate, and testes). A second group observed 5-ht_6 mRNA signals in the hippocampus, hypothalamus, adrenal, and stomach (7). Initial in situ hybridization studies in the rat brain demonstrated high levels of mRNA in the striatum and olfactory tubercles (7). Other labeled structures included the nucleus accumbens, olfactory bulb, and hippocampus. Using in situ hybridization, Ward et al (38) have completed a detailed examination of the mRNA distribution for the 5-ht_6 receptor in the rat brain. Their study confirmed the high abundance of message in the olfactory tubercle, striatum, nucleus accumbens, dentate gyrus, and CA1, CA2, and CA3 fields of the hippocampus. Lower intensity labeling was found in the cerebellum, some diencephalic nuclei, amygdala, and several cortical layers (layers 2, 3, 4, and 6). In the striatum, the 5-ht_6 mRNA is extensively co-localized with enkephalin (68%), substance P (79%), and dynorphin (59%) output (39). Similar co-localization was detected in the substantia nigra. In the striatum, the 5-ht_6 transcripts are homogeneously distributed between the patch and matrix components as well as between cells projecting to the two major outflow pathways.

The distribution of the human 5-ht$_6$ mRNA has been evaluated by Northern blot analysis (8). It parallels the distribution shown in the rat brain, with the highest expression levels detected in the caudate. In the human brain, lower expression levels of 5-ht$_6$ mRNA were found in the hippocampus, amygdala, and thalamus.

Localization of the 5-ht$_6$ Receptor Protein

Immunohistochemistry The pattern of protein expression for the 5-ht$_6$ receptor has been determined using selective antibodies to a carboxyl-terminal domain of the receptor sequence (27). Receptor protein was abundant in the plexiform layer of the olfactory tubercle and in the frontal and entorhinal corticies, nucleus accumbens, striatum, hippocampus (striata oriens and radiatum of CA1 and molecular layer of the dentate gyrus), and molecular layer of the cerebellum. A moderate degree of immunoreactivity was found in the thalamus, the substantia nigra, the superficial layer of the superior colliculus, the motor trigeminal nucleus, and the facial nucleus (27). This pattern is consistent with that seen from determination of the mRNA distribution, indicating that the protein is close to the site of synthesis, as in dendrites or somata. Dendritic localization in the striatum and dentate gyrus has been visualized by immuno-electronmicroscopy. The strong distribution of the receptor protein in the extrapyramidal and limbic areas led to the suggestion that the 5-ht$_6$ receptor may control motor function and mood-dependent behaviors. Gerard et al (27) further suggested that the 5-ht$_6$ receptors may be on the target cells of dopaminergic neurons (but see below), which might explain part of the antipsychotic activity of clozapine.

Autoradiography Radioligand binding is an alternative to immunohistochemistry to map receptor protein in the rat brain. The first experiment used [^{3}H]clozapine as a label for 5-ht$_6$ receptors in membranes (40). Forty percent of the sites that Glatt et al (40) detected exhibited a 5-ht$_6$ profile. There were no differences in the density of these sites between cerebral cortex, striatum, and hippocampus. The [^{3}H]clozapine binding is consistent with data from in situ hybridization studies. Future studies using [^{3}H]clozapine for receptor autoradiography could provide a detailed map of 5-ht$_6$ receptors. Methiothepin is a ligand that has even higher affinity for the 5-ht$_6$ receptor. It has previously been radiolabeled with tritium, but it has not been found to be a suitable radioligand in the brain (41), as a result of its physico-chemical properties (e.g. lipophilicity) and poor receptor subtype selectivity.

Recently, a new radioligand, [^{125}I]SB-258582, has been introduced that is selective for the cloned 5-ht$_6$ receptor (35). Subsequent studies (37) indicate that there is high specific binding in native tissues and that this ligand is useful for autoradiographic mapping studies. In rat, high densities of sites were found in the cerebral cortex, nucleus accumbens, caudate-putamen, and CA1 and dentate gyrus of the hippocampus. A moderate density of labeling was detected in the thalamus

and substantia nigra. Furthermore, after lesioning with 6-hydroxydopamine (6-OHDA) to the median forebrain bundle, no changes in the levels of binding were found, although there was a complete loss of tyrosine hydroxylase immunoreactivity in the striatum and nigra. This indicates that the 5-ht_6 receptors may be on cholinergic or GABAergic interneurons in the caudate putamen or on striatal GABAergic neurons or on their terminal fields in the nigra.

c-fos Activation Neuronal activation had been monitored using antibodies to the immediate early gene c-fos after drug treatment. Typical and atypical antipsychotic compounds give characteristic distribution patterns of c-fos activation (42, 43). The high affinity of antipsychotic compounds at the 5-ht_6 receptor implies that part of their actions may be due to their action on this receptor. The selective 5-ht_6 antagonist SB-271046 was administered to rats for four days, and the brains were processed for c-fos immunoreactivity (44). Rats treated with clozapine or haloperidol were run in parallel for comparison. Clozapine enhanced c-fos levels in the median prefrontal cortex and nucleus accumbens, and haloperidol enhanced levels in the caudate putamen and nucleus accumbens. No enhancement was seen in the SB-271046–treatment group (although the caudate putamen was not examined). These data indicate that activity of clozapine as monitored in this assay is not primarily the result of its action at the 5-ht_6 receptor.

FUNCTIONS OF THE 5-HT_6 RECEPTOR

Cellular Responses

There are several early reports of "atypical" 5-HT receptors in cells lines, particularly NCB.20 cells. This cell line was created by fusing a mouse neuroblastoma line, N18TG2, and an embryonic hamster brain explant (45). In this cell line, 5-HT, 5-MeOT, and methysergide all stimulated cAMP production. Clozapine and spiperone were antagonists. This response was reinvestigated using the newer tools for characterization (46, 47). cAMP stimulation was inhibited by metergoline (K_b = 50 nM), but not by ICS 205–930 (47), consistent with a 5-ht_6 but not a 5-HT_4 or 5-HT_7 response profile. The parental mouse cell line (N18TG2) was also evaluated (17). 5-HT stimulates cAMP responses with a pharmacological profile similar to that of the cloned 5-ht_6 receptor. The rank order of agonist potency in both radioligand binding and second messenger assays was 5-MeOT > 5-HT > tryptamine > 2-Me-5-HT >> 5-CT > α-Me-5-HT. In binding assays methiothepin showed higher affinity than clozapine, while in second-messenger assays the antagonists methiothepin, clozapine, and mianserin exhibited similar potencies (pA_2 = 6.5). A molecular analysis of the N18TG2 cell line to evaluate the presence of mRNA for 5-HT receptor subtypes has not been reported.

In primary neurons, stimulatory AC responses mediated via a 5-ht$_6$–like receptor have also been described (48). In cultured mouse striatal neurons, the rank order of agonist potencies to stimulate cAMP production was 5-HT > LSD > 5-MeOT > 5-CT. The serotoninergic agonists 8-OH-DPAT, sumatriptan, and cisapride were inactive. This response was antagonized by methiothepin, nortriptyline, clozapine, and amitriptyline. In combination with high distribution of mRNA in the striatum, this pharmacological profile indicates that these were 5-ht$_6$ responses in native neurons.

Furthermore, mRNA for 5-ht$_6$ receptors has been detected in an immortalized serotoninergic cell line from rat raphe nuclei (49). This may serve as an interesting model system for future studies of 5-ht$_6$ receptor regulation in a neuronal context.

TISSUE RESPONSES

Potential functional correlates of 5-ht$_6$ receptors have also been observed in vitro (50). A study of glycogenesis in tissue slices from rat cortex may reflect a 5-ht$_6$–like profile. In this preparation, 5-HT, 5-MeOT, and tryptamine stimulated glycogen hydrolysis. Tricyclic antidepressants were among the most potent competitive antagonists of the response. Methiothepin was weaker than expected for a 5-ht$_6$ response in antagonizing the glycogen hydrolysis response; physicochemical properties of the compound may have limited its efficacy. N,N-dimethyltryptamine (N,N-DMT) also antagonized this response, but its efficacy was greater than methiothepin. At the human 5-ht$_6$ receptor, N,N-DMT was equipotent with 5-HT (pK_i = 7.2).

In pig caudate membranes (51), a rank order of agonist potencies similar to that determined for 5-ht$_6$ was observed: 5-HT = 5-MeOT > 5-CT. The agonists 8-OH-DPAT, sumatriptan, and renzapride were inactive. The antagonist rank order was methiothepin > clozapine >> ketanserin. Neither of these receptor profiles derived from striatal preparations exactly matches the rank order of potencies in the N18TG2 cell line or the rank order of binding affinities from the cloned rat receptor. However, cross species comparisons or methodological differences may obscure the true relationships.

Electrophysiology

At present, there are no available reports of electrophysiological studies on the 5-ht$_6$ receptor.

IN VIVO STUDIES OF 5-HT$_6$ RECEPTOR FUNCTION

Molecular Approaches to Function In Vivo

Antisense Oligonucleotides The first behavioral studies of possible 5-ht$_6$–mediated function have been attempted using antisense oligonucleotides (AOs) targeted to the 5-ht$_6$ receptor subtype (52). In these studies, the rats exhibited a

behavioral phenotype consisting of an increased number of yawns and stretches. This behavior was blocked by atropine, suggesting a role of the 5-ht$_6$ receptor in the control of cholinergic neurotransmission. If so, then a 5-ht$_6$ antagonist might be useful in the treatment of depression, anxiety, and/or memory disorders (52).

Using a similar approach, Yoshioka and colleagues (53) evaluated the effect of AOs in a conditioned fear stress paradigm (CFS). After seven days of AO administration to the lateral ventricle, the 5-ht$_6$ receptor number decreased by 30%. In these animals, but not the sense oligonucleotide controls, the CFS-induced 5-HT release was suppressed, although freezing behavior was unaffected. This result suggests a potential role for the 5-ht$_6$ receptor in some forms of anxiety.

Finally, a preliminary report has appeared linking the 5-ht$_6$ receptor to memory acquisition and feeding (54). Again using AOs applied i.c.v., rats were treated for six days and evaluated in the Morris water maze test. AO-treated rats had no differences in visual acuity or swim speed, but they had a shorter average latency and longer time spent on the learned platform than controls. In addition, they had a lower body weight. Confirmation of these fascinating results is awaited.

Knockout Mice Targeted gene disruption has served as a useful probe for receptor function (55). A constitutive knockout animal lacking functional 5-ht$_6$ receptors has been produced and evaluated in several tests. At present, the only detectable difference from the wild-type animals has been an increase in anxiety-like behavior in the elevated zero maze (56). Additional studies are required to fully probe the changes in behavior and physiology in this knockout mouse.

Selective Antagonist Studies of 5-ht$_6$ Receptor Function

The selective 5-ht$_6$ receptor antagonist Ro 04–6790 was administered to rats by systemic injection. The compound induced a behavioral syndrome that included a dose-dependent increase in yawning, stretching, and chewing and was similar to that seen with the antisense treatment (32). The maximal effect was obtained at a dose that gave a cerebrospinal fluid concentration sufficient to occupy more than 70% of the 5-ht$_6$ receptors. Further exploration of this syndrome revealed that the stretching component was dose dependent and statistically significant (57). Pretreatment with muscarinic antagonists inhibited the stretching induced by Ro 04–6790. A non-CNS penetrant muscarinic antagonist was unable to inhibit the behavior, indicating a central mechanism. In addition, haloperidol had no effect. As with the antisense treatment, the 5-ht$_6$ antagonists produced a stretching behavior that is likely to be mediated by an increase in cholinergic, but not dopaminergic, neurotransmission. In contrast, the yawning was neither dose dependent nor statistically significant. This was in contrast to the effect of AO treatment on yawning. A number of explanations are possible, but further studies are required to evaluate them.

The distribution and pharmacology of the 5-ht$_6$ receptor suggest a link with dopaminergic function. mRNA for the 5-ht$_6$ receptor is preferentially down-

regulated in rats in certain brain regions after a two-week treatment with clozapine or haloperidol (58). Bourson and colleagues (59) investigated the effects of Ro 04–6790 on dopaminergic function. Ro 04–6790 did not induce catalepsy and had no effect on haloperidol or SCH 23390–induced catalepsy. It did not elicit rotational behavior in rat with unilaterally lesion of the median forebrain bundle induced by 6-OHDA. Ro 04–6790 had no effect on L-Dopa or amphetamine-induced rotational behavior. In contrast, antagonism of the 5-ht$_6$ receptor inhibited rotational behavior in the lesioned rats in response to cholinergic antagonists such as scopolamine and atropine. Therefore, consistent with previous reports using oligonucleotides, 5-ht$_6$ receptors are involved in cholinergic but not dopaminergic neurotransmission.

Using a second and more highly brain penetrant 5-ht$_6$ antagonist, Routledge et al (60) demonstrated that SB-271046 significantly potentiated physostigmine-induced yawning. It was also tested in two models of cognition enhancement (61). SB-271046 improved retention in the water maze test of spatial learning and memory. The compound also produced a significant improvement in performance of aged rats in an operant-delayed alternation task. These results all suggest that the 5-ht$_6$ receptor is implicated in the control of central cholinergic function and may be an interesting avenue for the treatment of cholinergic defects in cognitive dysfunctions such as Alzheimer's disease. Taken in the context of the 6-OHDA lesioning studies along with autoradiography, functional 5-ht$_6$ receptors may be on cholinergic or GABAergic interneurons in the caudate putamen or on striatal GABAergic neurons or their terminal fields in the nigra. This distribution is consistent with the proposal that 5-ht$_6$ receptors may regulate motor function and control memory and mood (37).

POTENTIAL THERAPEUTIC INDICATIONS FOR 5-HT$_6$ RECEPTORS

The distribution of the 5-ht$_6$ receptor, as well as its affinity for antipsychotic compounds, has led to significant efforts to understand its possible role in psychiatry. Mapping and lesioning studies so far indicate that there is no direct involvement of 5-ht$_6$ receptors in dopaminergic neurotransmission. However, two genetic association studies have been recently reported with respect to the 5-ht$_6$ receptor gene. The first looked at association between the 5-ht$_6$ receptor gene and schizophrenia (62) in a Japanese population. Three hundred subjects were genotyped for the biallelic variation (267C/T); half were schizophrenic and half were healthy controls. No significant difference in allele frequencies was detected between the schizophrenic patients and the healthy controls. This suggested that the 5-ht$_6$ receptor gene may not contribute directly to schizophrenia. However, a second study evaluated the relationship between the C267T polymorphism and the clinical response of schizophrenic patients, who were refractory to typical

antipsychotics and to the atypical antipsychotic compound clozapine (63). Ninety-nine chlorpromazine-resistant patients of the same ethnic Chinese background were genotyped and their response to clozapine after a minimum of eight weeks was determined. The distribution of the three possible genotypes was in Hardy-Weinberg distribution. There were no differences in baseline scores. In 60.6% of patients, the Brief Psychiatric Rating Score (BPRS) decreased by over 20% from baseline after clozapine treatment. Patients with the 267T/T genotype had a significantly better response to clozapine than the other two groups. Although the group size was small, the results were significant. The changes in general symptoms were also close to significance. This parameter reflects somatic concern, anxiety, guilt, tension, and depressed mood, i.e. the emotional control systems. These results are particularly interesting since C267T is a silent mutation that does not change the amino acid sequence. It may, however, affect parameters such as RNA stability or translational efficiency. Although a larger study is needed to confirm these observations, they may suggest that the 5-ht_6 genotype may help predict patients' responses to clozapine.

A surprising outcome of the antisense studies, confirmed by the antagonist experiments, is the role of 5-ht_6 receptors in the control of central cholinergic function (59, 61). This is also supported by localization and lesion studies. Although the antagonist data have appeared in preliminary form only, it is exciting that the 5-ht_6 antagonists may have a role in the treatment of cognitive dysfunction. Other possible avenues presently under investigation are the link to depression and anxiety (53) and the effect on body weight (54). As the new pharmacological tools become more widely available, the larger picture of 5-ht_6 receptor function will be sketched.

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